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Full Length Research Paper

Impact of cassava starch-alginate based coatings added with ascorbic acid and elicitor on quality and sensory attributes during pineapple storage

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Pineapple is a tropical fruit of great consume demand. However, due to its high perishability at room conditions, there is noticeable loss of quality postharvest in a short period. In this context, the use of biodegradable coatings is a promising alternative for maintaining postharvest quality. Thus, the aim of this study was to evaluate the use of cassava starch-alginate based biodegradable coatings added with ascorbic acid and an elicitor on postharvest quality and conservation of 'Pérola' pineapple. Fruits were coated with: Cassava starch 1.5% + alginate 0.5%; cassava starch 1.5% + alginate 0.5% + ascorbic acid 0.18 %; cassava starch 1.5% + alginate 0.5% + elicitor, each added of 0.5% of glycerol as a plasticizer, and the control (uncoated fruits), following storage at room conditions (23 ± 1°C, 88 ± 2% RH). Pineapples coated with cassava starch-alginate associated with the elicitor (SE) or ascorbic acid (SA) kept lower levels of reducing and total sugars as well as better appearance and general acceptance (GA) during room storage for 18 days. According to the panelists, the determining factors for higher GA of 'Pérola' pineapple under these coatings were the sweetness, fresh like characteristics of taste and odor, and better appearance. Overall, the use of SE and SA coatings had a marked impact in maintained the quality during 18 days and did not adversely affect the sensory characteristics of 'Pérola' pineapple stored at room conditions.

Key words: *Ananas comosus, Manihot esculenta,* biodegradable coating, sugars, sensorial acceptance.

INTRODUCTION

Pineapple (*Ananas comosus* var. comosus) stands out as one of the most important fruit crops in tropical and subtropical countries (Paull and Chen, 2003), especially in Brazil where it is widely cultivated, producing 1,762,938 fruits in 2014, ranking among the three largest world producers (IBGE, 2016). Despite its extensive production in Northeastern Brazil, the pineapple chain has several critical constraints that have limited its expansion to new markets, even inside the country (Martins et al., 2012). In this context, postharvest losses

are the main concern, which are related to its high perishability that contributes to its short postharvest life (Silva et al., 2010), and especially those related to physiological disorders such as internal browning (Luengwilai et al., 2016). Additionally, exposure to oscillating ambient conditions during marketing results in decreasing its overall quality, indicating that the pineapple fruit chain demands more innovation (Dantas et al., 2015) in order to maintain postharvest fruit quality and acceptance by consumers (Hounhouigan et al., 2014). This is especially true in the context of the production systems in the Northeastern Brazil, which are characterized by low income producers and conventional practices of crop management (Dantas Junior et al., 2009).

Pérola cultivar is the most consumed pineapple in Brazil (Dantas et al., 2015). However, it presents a short postharvest life (Martins et al., 2012). Some efforts have been directed to extend postharvest conservation using cold storage and associated technologies such as plant regulators (Zhang et al., 2015), modified atmospheres (Chiumarelli and Hubinger, 2014), and polysaccharide-based coating (Lima et al., 2012). However, these approaches need to be optimized in terms of improving the barrier properties of the coatings (Dhall, 2013).

The use of biodegradable coatings have been reported as a way to prolong the postharvest life of whole fruit (Azerêdo et al., 2016) and fresh-cut products (Bitencourt et al., 2014). Due to their specific properties, coatings act as good barriers to gases (Hamzah et al., 2013), resulting in decreased respiratory rate (Chiumarelli et al., 2011), and reduced water loss (Lima et al., 2012). Furthermore, incorporating active compounds in the polymer matrix such as essential oil found in chitosan (Aloui et al., 2014) and in starch-based coating (Oriani et al., 2014), can assist in controlling diseases and reducing metabolic rate, thus enhancing postharvest life and adding value to the product through exploiting safe, sustainable, and affordable local raw material.

More recently, the development of functional coatings has been discussed, which depend on the intrinsic properties of the matrix and the embedded materials, as well as the type of fruit regarding its postharvest life and quality maintenance. However, an important aspect to be noted is that the use and application of coatings are ruled by the laws of the country in which it is being applied, and/or the country to which the fruits are to be exported (Dhall, 2013). Thus, incorporating active components in coatings has attracted the attention of researchers, since it is supposed to modulate either the fruit's physiology or be adjusted to the matrix, changing its properties. In this context, elicitors are compounds which activate chemical defense in plants and have been used for disease control (Thakur and Sohal, 2013), and can be useful for postharvest applications. Different types of elicitors have been characterized, including inorganic compounds, carbohydrate polymers, lipids, glycopeptides, and glycoproteins (Terry and Joyce, 2004). However, many more studies are needed about the impact of coatings composed of widely available raw material such as cassava starch on postharvest quality of pineapple, in which the matrix is embedded with functional ingredients (Ghidelli et al., 2014).

Starch from cassava (*Manihot esculenta*) seems to be a promising raw material to develop coatings due to its physical properties and workability, allowing combining with other components to improve its mechanical properties and form stable emulsions if lipids and hydrocolloids are combined (Santos et al., 2014). In this context, alginate has been successfully used as a component of the polymeric matrix of coatings (Chiumarelli et al., 2011), and glycerol as a plasticizer (Dhall, 2013). In addition, edible coatings and films may also act as food additive carriers, including antioxidants and antimicrobial compounds (Jiménez et al., 2012). Even though the pineapple has an irregular surface, application of a polysaccharide-based coating may be an affordable option to reduce its postharvest losses and maintain quality under room conditions. Thus, the aim of this study was to evaluate the use of cassava starch-alginate based biodegradable coatings added with ascorbic acid and elicitor in the postharvest conservation of 'Pérola' pineapple.

MATERIALS AND METHODS

Fruit harvest

Fresh pineapple (*Ananas comosus* var. comosus) fruits were harvested in the commercial maturity (beginning of yellow pigmentation at the fruit base) from an orchard located at the municipality of Santa Rita, State of Paraíba, Brazil. Fruits were selected with weight ranging between 0.9 and 1.2 Kg, presenting uniform size and regular shape without visible defect. Pineapples were transported to the Postharvest Biology and Technology laboratory of the Centro de Ciências Agrárias, of the Universidade Federal da Paraíba, Brazil, to be evaluated. Fruits were manually washed, then immersed in a 200 mg/L sodium hypochlorite solution for 5 min, and immersed in distilled water for 2 min. After drying at room conditions, pineapples were separated into four groups for the application of coatings.

Preparation and coatings application

Three coating polymeric matrixes were designed. Initially, 1.5%

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Table 1. Formulations for cassava starch-alginate based coatings applied in 'Pérola' pineapple harvested at the commercial maturity.

Coating code	Cassava starch (%)	Sodium alginate (%)	Glycerol (%)	Additive
C	-	-	-	-
SA	1.5	0.5	0.5	Ascorbic acid (0.18 %)*
SE	1.5	0.5	0.5	Elicitor (0.4 %)
S	1.5	0.5	0.5	-

*Ghidelli et al. (2014).

cassava starch dispersion was prepared by gelatinization of the starch, which consisted of heating the solution to 70°C under constant stirring (Lima et al., 2012). Following the gelatinization of the cassava starch, the additional components and additives were added under constant stirring until the complete homogenization (Table 1). All coating dispersions were added with 0.5% sodium alginate (Sigma-Aldrich) and 0.5% glycerol (Sigma-Aldrich), as a plasticizer. The formulation cassava starch-alginate + ascorbic acid (SA), was added with 0.18% ascorbic acid (Sigma-Aldrich). The formulation cassava starch-alginate + Elicitor (SE) was added with 0.4% of the elicitor. Uncoated pineapples were the control treatment (C).

Pineapples were immersed in each cassava starch-alginate based coating for 1 min under a fruit smooth rotation for better adherence of dispersion. Then, coated fruit was kept at room conditions until complete drainage. Afterward, pineapples were placed in styrofoam trays and stored under room conditions (23 ± 1°C and 88 ± 5% RH) for 18 days. The elicitor was composed by bioflavones, phytoalexins, and polyphenols (1.66 g/100 mL), ascorbic acid (1.65 g/100 mL), lactic acid (0.95 g/100 mL), citric acid (1.30 g/100 mL), and vegetable glycerin (6.60 g/100 mL) (Ecolife® QUINABRA - Química Natural Brasileira Ltda., Sao Paulo, Brazil).

Experimental design

The experiment was performed in a completely randomized design in a 4 × 4 factorial scheme, combining 4 coatings (S, SA, SE, and C) and 4 evaluation periods (0, 6, 12, and 18 days), using four replications, consisting of 6 pineapples each. However, the sensory analysis had a randomized block design, with the same factorial scheme, where the 24 trained panelists were considered the replications.

Physical and physiochemical evaluations

Firmness was measured with digital penetrometer (Magness Taylor Pressure Tester, Canada) at two equational regions of each fruit at different storage time using a 6 mm diameter probe and results were expressed in Newton-N. Weight loss was measured by recording the fruit weight during the storage time. The percentage of weight loss was relative to the initial value (taken as 0%) (Martins et al., 2012).

The following physicochemical characteristics were determined according to AOAC (2012): The content of soluble solids was determined with a digital refractometer with automatic temperature compensation (model ATAGO N1) and expressed as percentage; acidity was determined by titration with 0.1 M NaOH and was expressed as gram of citric acid per 100 g of fresh weight (fw); SS/TA *ratio* was obtained by the relation between the soluble solids and titratable acidity; pH was measured from the acidity extract before titration with a pH-meter; the reducing, non-reducing, and total sugars (g/100 g fw) were determined by titration with Fehling's solution.

Sensory evaluations

For the sensory characteristics evaluation, twenty four panelists, regular pineapple consumers, were selected between 18 and 40 years old. The panelists were trained based on the perception of the acidity (AC), sweetness (SW), characteristic taste (CT), characteristic odor (CO) of the samples based on 5-point hedonic scales (9-very intense, 7-intense, 5-moderate, 3-light, and 1-absent). For the off odor (OO) and off taste (OT), a structured scale varying from 1 to 6 (6 = Absent; 3 = Moderate; 1 = Strong) was used to express the degree of unacceptability (Mascarenhas et al., 2010). The 5-point hedonic scale for color (CL), considering the fruit skin color, was 1 (100% green and 0% yellow), 2 (75% green and 25% yellow), 3 (50% green and 50% yellow), 4 (25% green and 75% yellow) and 5 (0% green and 100% yellow). The hedonic structured scale varying from 1 to 9 (9 = Liked very much; 5 = not like or dislike; 1 = Disliked very much) was used to express the degree of acceptability for appearance (AP) and general acceptance (GA) (Miguel et al., 2010). Sensory tests were carried out in morning sessions at sensory laboratory equipped with individual sensory cabinets. The panelists used water and salted cracker as palate cleanser between one sample to another, respecting a 1 min rest time among the samples evaluations. The samples for taste proofs were placed in plastic cups at room conditions (23 ± 1°C and 88 ± 5% RH), codified with three-digit number codes, and in a randomized and balanced order of sample presentation. Scores and comments of the panelists were recorded on scorecards.

Statistical analysis

Data were submitted to analysis of variance by F test ($p \leq 0.05$). For the storage period (days), the polynomial regression analysis was applied, testing up to cubic level and coefficient of determination higher than $R^2 > 0.5$. Additionally, treatments (coatings) were individually analyzed by the Tukey test ($p \leq 0.05$) for each day at room storage when regression did not meet the required criterions. Multivariate analysis, such as principal components analysis (PCA) and Ward's clustering were performed to correlate the variables that have been more affected by the applied coatings during storage. Statistical analysis were performed with the Sisvar 5.6 software (Ferreira, 2008) and JMP v10.0.0 (SAS©, 2012).

RESULTS

Firmness decreased during storage, but was maintained higher for 15 days in coated pineapples. For uncoated pineapples, firmness reduced nearly 25% by the 12th day of storage (Figure 1A). In fact, uncoated pineapples (C)

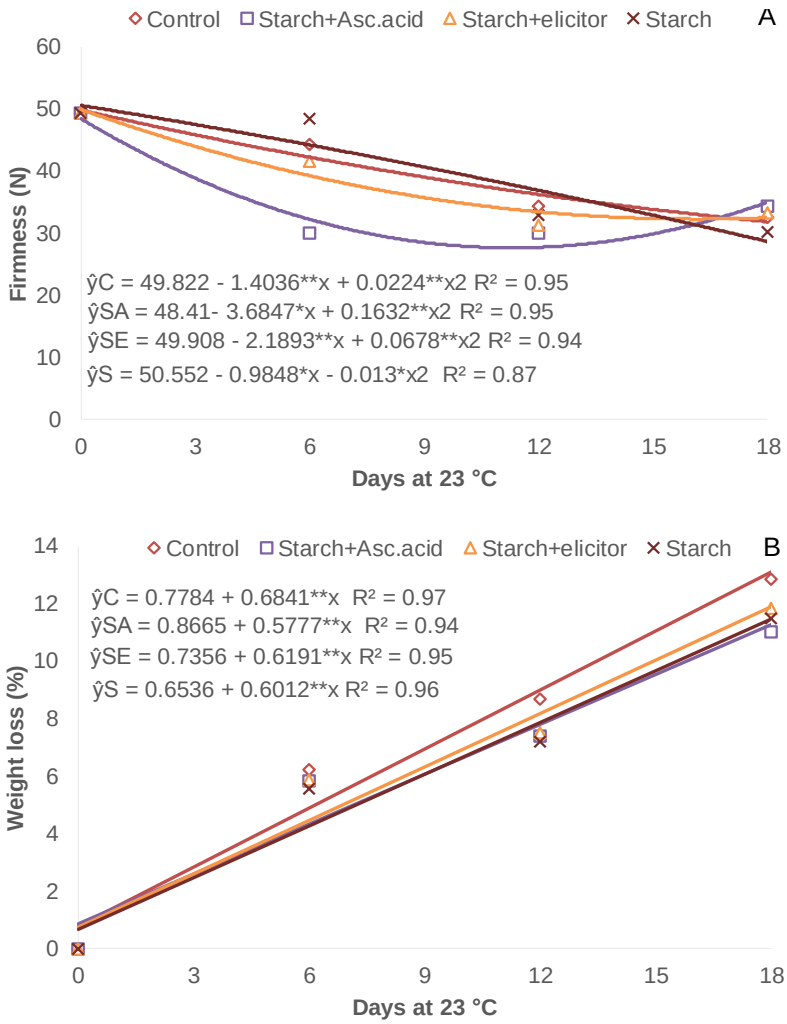

Figure 1. Firmness (A) and weight loss (B) in 'Pérola' pineapple, coated with cassava starch-alginate (S), cassava starch-alginate + ascorbic acid (SA), cassava starch-alginate + elicitor (SE), and uncoated fruit (C), during 18 days storage at room conditions (23 ± 1°C and 88 ± 5% RH). n=4.

had the highest weight loss during storage with 12.86% at 18 days of storage. In turn, starch-alginate based coatings (S, SA, and SE) presented lower weight losses (Figure 1B). Indeed, the highest slope was obtained for pineapples from the Control group (0.68). Soluble solids (SS) content was affected by the application of cassava starch-alginate based coatings. SS content was higher for the uncoated pineapples (C) and lower in pineapples coated with cassava starch-alginate (S) throughout storage (Table 2). In turn, fruits coated with starch-alginate + ascorbic acid (SA) and starch-alginate + elicitor (SE) showed intermediate levels for SS during the 18 days of storage, indicating a lower metabolic rate provided by these coatings to 'Pérola' pineapples, mainly for the SA. Titratable acidity (TA) in 'Pérola' pineapples did not differ among coatings until the 12th day of storage. However, pineapples coated with cassava starch-alginate (S) showed a decline in the TA content afterward (Table 2), which was followed by a higher pH. However, the pH of the pulp of pineapples from other coatings did not differ much throughout the 18 days of storage. The observed decline in TA in S-coated pineapples during the storage provided the highest values for the SS/AT ratio.

The initial mean content of reducing sugar (RS) was 2.27 g/100 g, which declined during the 18 days of storage. This reduction in the RS was much faster for S-coated pineapple until the 15th day of storage. In turn, pineapples coated with starch-alginate + elicitor (SE) and starch-alginate + ascorbic acid (SA) showed the highest RS content throughout storage (Figure 2A). Non-reducing sugar (NRS) content declined until 9 days of storage, and increased thereafter, regardless of the coating applied to the pineapples (Figure 2B). However, this decline was much lower for uncoated pineapples (C), and, in turn, faster for coated pineapples, mainly for the S-coated ones. After 9 days of storage, pineapples coated with

Table 2. Soluble solids (SS), titratable acidity (TA), SS/TA ratio, and pH in 'Pérola' pineapple coated with cassava starch-alginate (S), cassava starch-alginate + ascorbic acid (SA), cassava starch-alginate + elicitor (SE), and uncoated fruit (C) during 18 days storage under room conditions (23 ± 1 °C and 88 ± 5% RH).

Days		Soluble solids (%)	Titratable acidity (g.100g^{-1})	SS/AT Ratio	pH
0	C	11.17^{a}	0.75^{a}	14.89^{a}	3.76^{a}
	SA	11.17^{a}	0.75^{a}	14.89^{a}	3.76^{a}
	SE	11.17^{a}	0.75^{a}	14.89^{a}	3.76^{a}
	S	11.17^{a}	0.75^{a}	14.89^{a}	3.76^{a}
6	C	12.83^{a}	0.85^{a}	15.09^{a}	3.74^{b}
	SA	11.50^{b}	0.79^{a}	14.57^{a}	3.85^{ab}
	SE	12.25^{a}	0.85^{a}	14.43^{a}	3.89^{a}
	S	10.50c	0.82^{a}	12.86^{b}	3.85^{ab}
12	C	12.33^{a}	0.84^{b}	14.62^{a}	3.72^{a}
	SA	12.75^{a}	0.91^{a}	13.96^{a}	3.75^{a}
	SE	12.58^{a}	0.82^{b}	15.33^{a}	3.73^{a}
	S	11.67^{b}	0.80^{b}	14.63^{a}	3.77^{a}
18	C	13.00^{a}	0.87^{a}	14.88^{ab}	3.84^{b}
	SA	11.73^{b}	0.84^{a}	14.02^{b}c	3.83^{b}
	SE	11.33^{b}c	0.86^{a}	13.23c	3.94^{ab}
	S	10.97c	0.68^{b}	16.08^{a}	4.05^{a}

Means followed by the same letter in the column do not differ by Tukey's test ($p \leq 0.05$).n=4.

only cassava starch-alginate (S) had the lowest levels of sugars (4.47 g/100 g), which were kept lower throughout storage. Total sugar (TS) content decreased until the 12th day of storage. However, regarding NRS, TS was higher for uncoated (8.66 g/100 g) and lower for S-coated pineapple (4.45 g/100 g) from the 12th day of storage (Figure 2C).

Sensory attributes of 'Pérola' pineapple under the different coatings are shown in Table 2. According to the panelists, pineapple acidity (AC) is a characteristic feature of the acceptance of this cultivar, and was reduced over time; mainly for the S-coated pineapples from the 12th day onward. During the 18 days of storage, perception of acidity was reduced by 65%, which negatively impacted general acceptance. In turn, the sweetness (SW) perception of pineapple pulp did not differ ($p \leq 0.05$) among the coatings applied throughout storage.

Scores for characteristic taste (CT) decreased during storage, notably for S-coated pineapple, with the lowest score of 3.47 (light) during the 18 days of storage. In turn, the characteristic odor (CO) was maintained until the 12th day, regardless of the coating applied. However, the CO decreased for uncoated and S-coated pineapples during the room storage. The off taste (OT) was not reported by the panelists until the 12th day. However, the OT later reached the acceptance limit (score 4) for uncoated pineapples as well as for S-coated fruits, which was reported by the panelists as tasteless. The same pattern was observed for the off odor (OO), where uncoated pineapples reached 4.33 (close to the limit of acceptance) and S-coated pineapples reached 3.75 (below the acceptance limit) during the 18 days of storage. This was described by the panelists as presenting a fermented fruit smell, negatively impacting the CO, CT and, consequently, the GA. In turn, OT or OO were not reported for SA- or SE-coated pineapples by the panelists during the 18 days of room storage, with scores much above the acceptance limit, and without significant difference between these two coating formulations (Table 2).

The pineapple color evolved from green to orange during storage (Table 3; Figure 3), mainly for uncoated fruits, indicating the impact of coatings on retaining color development. However, SA- and SE-coated pineapples had the highest scores for appearance by the panelists, which characterized the fruits as fresh-like and with a turgid surface, while also presenting low brightness and a yellow color, absence of blemishes or diseases, severe damage and/or rot during the 18 days of storage. In turn, 'Pérola' pineapples coated with only starch (S) and the uncoated pineapples (C) had lower appearance scores than SA and SE after 12 days, being below the acceptable limit (5 – moderate). For uncoated pineapples (C), the observed decline in appearance during the 18 days of storage coincided with the color development and the transition from green (score 2) to yellow (score 5) (Table 3).

The general acceptance (GA) scores decreased during storage. However, they were kept higher for SA- and SE-coated pineapples during the 18 days of room storage. In turn, S-coated and uncoated pineapples (C) had scores

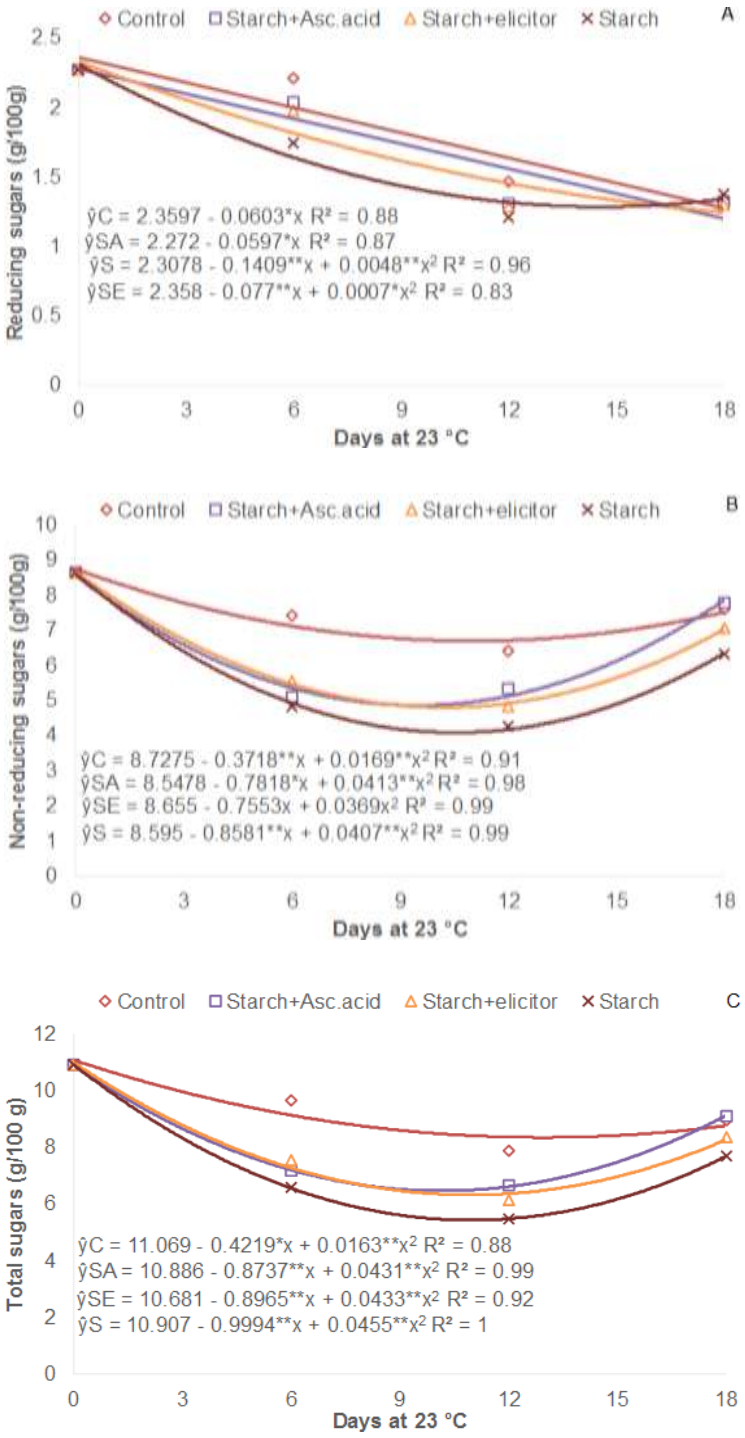

Figure 2. Reducing (A), non-reducing (B) and total sugars (C) in 'Pérola' pineapple coated with cassava starch-alginate (S), cassava starch-alginate + ascorbic acid (SA), cassava starch-alginate + elicitor (SE), and uncoated fruit (C) stored for 18 days under room conditions (23 ± 1°C and 88 ± 5% RH). n=4

below the general acceptance limit of 5.0 (5 - neither liked nor disliked). SA- and SE-coated pineapples presented good appearance, low shriveling and a suitable color at the end of the storage at room conditions (Figure 3).

Principal component analysis (PCA) covered 88.41% of

Table 3. Sensorial attributes for 'Pérola' pineapple (pulp and whole fruit) coated with cassava starch-alginate (S), cassava starch-alginate + ascorbic acid (SA), cassava starch-alginate + elicitor (SE), and uncoated fruit (C) stored for 18 days under room conditions (23 ± 1 °C and 88 ± 5% RH).

Days		Pulp						Whole fruit		
		AC	SW	CT	OT	CO	OO	CL	AP	GA
0	C	7.00^{a}	6.50^{a}	6.33^{a}	6.00^{a}	5.67^{a}	6.00^{a}	2.00^{a}	9.00^{a}	8.33^{a}
	SA	6.67^{a}	6.50^{a}	6.33^{a}	6.00^{a}	5.67^{a}	6.00^{a}	2.00^{a}	9.00^{a}	8.17^{a}
	SE	6.67^{a}	6.50^{a}	6.00^{a}	6.00^{a}	5.67^{a}	6.00^{a}	2.00^{a}	9.00^{a}	8.00^{a}
	S	6.67^{a}	6.50^{a}	5.67^{a}	6.00^{a}	5.67^{a}	6.00^{a}	2.00^{a}	9.00^{a}	8.00^{a}
6	C	6.67^{a}	6.67^{a}	7.33^{a}	5.33^{a}	6.00^{a}	6.00^{a}	3.67^{a}	6.33^{a}	7.83^{a}
	SA	6.33^{a}	6.50^{a}	6.33^{ab}	6.00^{a}	5.67^{a}	5.67^{a}	2.67^{a}	7.67^{a}	7.50^{ab}
	SE	6.67^{a}	6.83^{a}	5.67^{ab}	6.00^{a}	6.00^{a}	6.00^{a}	3.00^{a}	6.33^{a}	7.50^{ab}
	S	6.00^{a}	6.83^{a}	5.33^{b}	5.33^{a}	5.67^{a}	5.33^{a}	3.00^{a}	6.33^{a}	6.33^{b}
12	C	6.33^{ab}	6.83^{a}	5.67^{a}	4.00^{a}	6.00^{a}	5.67^{a}	4.67^{a}	5.00^{a}	6.67^{ab}
	SA	7.67^{a}	6.67^{a}	5.33^{a}	6.00^{a}	5.67^{a}	5.67^{a}	4.00^{a}	6.33^{a}	6.33^{ab}
	SE	5.67^{b}	7.00^{a}	5.67^{a}	5.33^{a}	5.00^{a}	5,67^{a}	4.33^{a}	6.33^{a}	7.50^{a}
	S	5.33^{b}c	7.00^{a}	4.67^{b}	5.33^{a}	5.67^{a}	5.33^{a}	4.33^{a}	5.00^{a}	6.17^{b}
18	C	6.67^{a}	6.83^{a}	5.33^{a}	4.00^{b}	3.67^{b}	4.33^{b}	5.00^{a}	4.00^{b}	4.17^{b}
	SA	4.67^{ab}	7.17^{a}	5.00^{a}	5.33^{a}	6.00^{a}	5.33^{a}	4.67^{b}	5.67^{a}	6.00^{a}
	SE	4.33^{ab}	7.33^{a}	5.00^{a}	5.00^{a}	5.67^{a}	5.00^{a}	4.67^{b}	6.33^{a}	6.50^{a}
	S	2.33^{b}	7.17^{a}	3.47^{b}	4.17^{b}	4.33^{b}	3.75c	4.67^{b}	4.00^{b}	4.00^{b}

Means followed by the same letter in the column do not differ by the Tukey's test ($p \leq 0.05$). n=24. AC, Acidity; SW, sweetness; CT, characteristic taste; OT, off taste; CO, characteristic odor; OO, off odor; CL, color; AP, appearance; GA, general acceptance. n=40. Scales: (AC, SW, CT and CO: 9-very intense, 1-absent; OO and OT: 6=absent, 1=strong; CL: 1=100% green, 5=100% yellow; AP and GA: 9=liked very much, 1=disliked very much). OF, off flavor; OT, off taste; Limit of acceptance = 4; AP, appearance; GA, general acceptance; Limit of acceptance = 5.

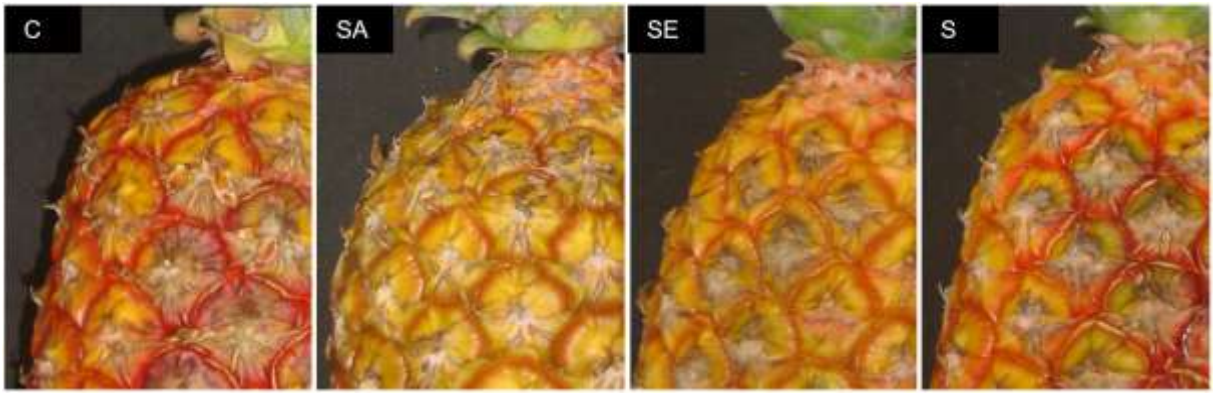

Figure 3. Appearance of 'Pérola' pineapple, coated with cassava starch-alginate (S), cassava starch-alginate + ascorbic acid (SA), cassava starch-alginate + elicitor (SE), and uncoated fruit (C), followed 18 days storage under room conditions (23 ± 1°C and 88 ± 5% RH).

the coatings variability, comprising two principal components, CP1 (61.30%) and CP2 (27.09%) (Figure 4A). The contribution of all analyzed variables regarding the two first principal components reflects the multivariate similarity of the grouped treatments, according to the overall responses of 'Pérola' pineapple to the imposed experimental conditions. Regardless of the variables SS/TA ratio, firmness, color, general appearance, and characteristic odor, which were significantly associated with PC2, all the physical, physiochemical, and sensory attributes explained the variability in PC1, meaning that the high correlation among these variables led the uncoated pineapple (Control) to differentiate itself, forming an isolated group.

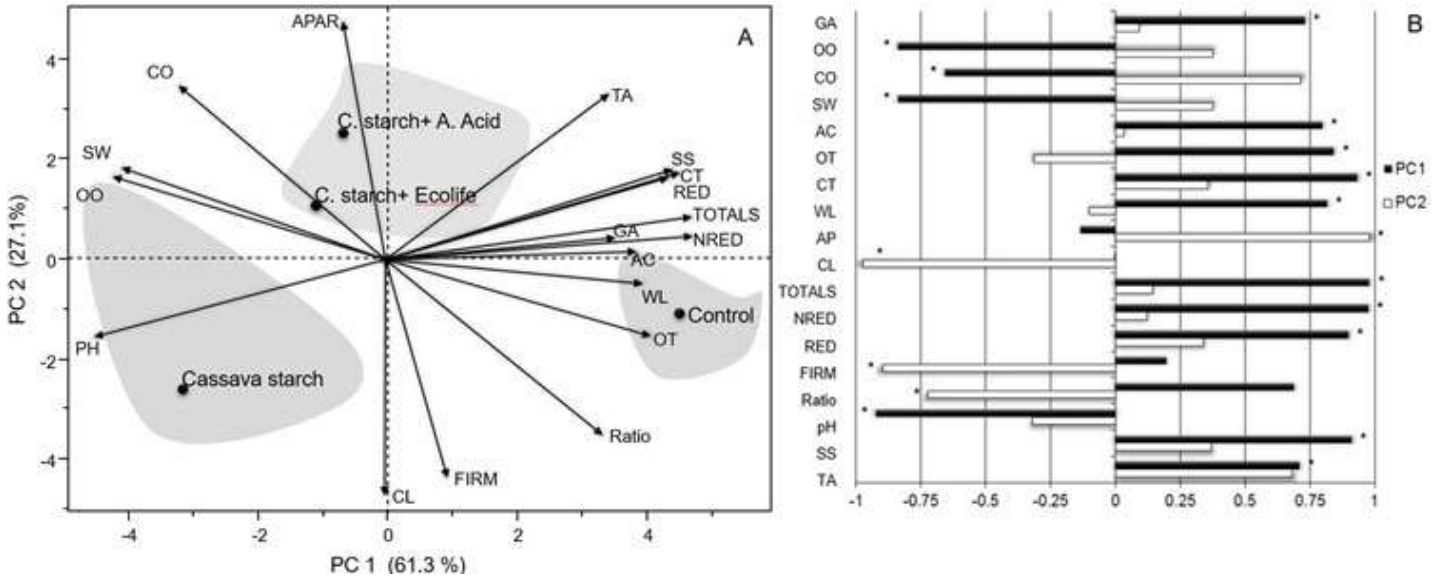

Figure 4. PCA plot of 'Pérola' pineapple quality attributes (physicochemical and sensorial) during storage and the plot of principal components correlations, PC1 and PC2. GA, General acceptance; CO, characteristic odor; OO, off odor; SW, sweetness; AC, acidity; CT, characteristic taste; OT, off taste; WL, weight loss; AP, appearance; CL, color; TOTALS, total sugars; NRED, non-reducing sugars; RED, reducing sugars; FIRM, Firmness; pH, SS/TA ratio ratio; SS, soluble solids; TA, titratable acidity of 'Pérola' pineapple coated with cassava starch-alginate S; SA, cassava starch-alginate + ascorbic acid; SE, cassava starch-alginate + elicitor; C, uncoated fruit stored for 18 days under room conditions (23 ± 1°C and 88 ± 5% RH). *The inclusion of significant variables was based on the multiplication of the highest score of each PC by 0.7, whose result was accepted as a threshold for inclusion.

Based on this, pineapple coated with cassava starch-alginate + elicitor (SE) and cassava starch-alginate + ascorbic acid (SA) were grouped together. For this group, pineapples of both coatings presented intermediate levels of titratable acidity, pH, soluble solids, reducing sugars, total sugars, and higher scores for characteristic taste and for general appearance. In turn, for starch-alginate-coated pineapple (S), the high PC scores for pH, firmness, off odor, and color separated it into another group. Interestingly, the highest PC scores for the physicochemical characteristics and the sensory attributes of off taste, acidity, low general acceptance, as well as higher weight loss led the uncoated pineapple to form a group and stay apart from the cassava starch coating group. In addition, the cassava starch-coated pineapples presented the lowest PC scores for pH and off odor perception by the panelists (Figure 4).

DISCUSSION

Reduction of firmness in pineapples was related to increased pectinesterase, polygalacturonase, and β-galactosidase activity (Rocculi et al., 2009). It has also been related to the coating composition, as polysaccharides are very hygroscopic and poor barriers to water vapor (Chiumarelli and Hubinger, 2014). Higher weight losses can also influence the water vapor transfer, resulting in increased fruit firmness in pineapples (Martins et al., 2012).

Therefore, coatings can be an alternative to minimize the weight loss of fruits and vegetables during postharvest storage (Plooy et al., 2009; Azerêdo et al., 2016), since they are able to efficiently control the gas exchange (Azarakhsh et al., 2014), being one of the main factors for increasing weight losses. Additionally, the coating components such as alginate play a noteworthy role in this effect, since they can deeply interfere with the mechanical properties of the coating (Chiumarelli and Hubinger, 2014), altering the barrier to water vapor and respiration gases (Azarakhsh et al., 2014).

The overall changes shown in the uncoated pineapples are correlated with the ambient conditions, especially temperature and humidity (Dantas Junior et al., 2009). However, there was a positive effect of the starch-alginate + ascorbic acid (SA) and starch-alginate + elicitor (SE) coatings in maintaining the soluble solids content of 'Pérola' pineapples compared with uncoated (C) or S-coated fruits, indicating that the addition of ascorbic acid or the elicitor can have an impact in reducing the metabolic rate. In general, pineapple do not present noteworthy changes in acid and sugars contents after harvested as they are a non-climacteric fruit, therefore they have no significant starch reserves that could be used in the metabolism, and then a slightly acid reduction is noticed in the postharvest (Paull and Chen, 2003).

Hong et al. (2013) reported that reducing sugar content

varied between 2 and 3 g/100 g during storage of summer pineapple, responding to temperatures and time in a manner that both RS and non-reducing sugars (NRS) had their content reduced by nearly 28% over time (0 - 24 days), and by 23% at different temperatures (6, 10 and 25°C).

As already well reported, NRS were much higher than RS content in pineapple (Martins et al., 2012), which herein were found in the proportion of 1:3.6 (NRS:RS). In this direction, Hong et al. (2013) reported a proportion of glucose and sucrose in the pulp in summer pineapple around 1:4.

Soluble sugars are important components of fruit quality, especially in pineapple, which is mainly composed of sucrose, glucose, and fructose (Hong et al., 2013). Higher sugar content in the uncoated pineapples (C) is possibly due to a higher metabolic rate, which is corroborated by higher SS and weight loss as a result of water loss and a possible concentration of the sugars. Therefore, coatings may act in attenuating the metabolic rate of 'Pérola' pineapples, especially those coated with SA and SE, which kept intermediate values of NRS and TS.

Higher acidity is one of the quality features that contribute to 'Pérola' pineapple preference by Brazilian consumers, in addition to being one of the main components of the pineapple taste which is balanced by the high sugar content (Berilli et al., 2011).

The addition of ascorbic acid or the elicitor improved the characteristics of the cassava starch-alginate film, impacting on the reduced metabolism of the 'Pérola' pineapples. Positive effects on fruit quality were also reported by Chiumarelli et al. (2011) for combination of carbohydrate polymeric matrix with antioxidants, and by Azerêdo et al. (2016) with plant extracts.

On the other hand, starch-alginate + elicitor (SE) and starch-alginate + ascorbic acid (SA) coated pineapples maintained the CO throughout storage, probably due to a positive effect of the ascorbic acid or the elicitor added to the polymeric matrix. Appearance is one of the main factors that influence consumers' purchase intentions (Azerêdo et al., 2016). Thus, coatings that are able to preserve the appearance of fruits are desirable for marketing.

In turn, color development was retained for SA- and SE-coated pineapple, and the scores for appearance were higher, indicating the efficiency of these coatings in keeping the fruit quality. GA evaluation was affected by all the previous sensory attributes, and reflected the panelists' perception about the overall quality as a trained judge and a consumer of pineapple, which enabled a more realistic judgement (Miguel et al., 2010). Importantly, the use of coatings postharvest would be efficient if they do not promote the buildup of off taste or off odor in the pulp, indicating that fruit quality maintenance can be achieved without affecting the sensory characteristics (Azarakhsh et al., 2014).

For SA and SE coatings, reduced permeability to oxygen and the combinations of the polymeric matrix with ascorbic acid and flavonoid elicitor provided a reduction in the metabolic rate (Ali et al., 2013), resulting in quality maintenance, and thus increasing the postharvest life. Uncoated pineapples continued their higher metabolism at room conditions, especially using the soluble sugars (Hong et al., 2013), resulting in faster decline in quality compared with the coated ones.

Conclusion

Overall, 'Pérola' pineapples coated with cassava starch-alginate added with ascorbic acid (SA) or elicitor (SE) had preserved sensory characteristics and weight loss minimized at 23°C, extending the postharvest life from 12 to 18 days. Furthermore, fruit coated with SE and SA presented lower levels of reducing and total sugars, which indicates a delay in ripening. These effects along with better appearance and general acceptance confirm the positive impact of cassava-alginate coating associated with antioxidants as a potential alternative for maintaining quality and enhancing the postharvest life of 'Pérola' pineapples maintained at room conditions. Thus, in 'Pérola' pineapples coated with SA and SE, maintenance of good appearance and characteristic taste, as well as lower weight loss were the main factors for the higher general acceptance.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Determination of macaw fruit harvest period by biospeckle laser technique

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Macaw palm has been stood out as a raw material for the production of bioenergy, because it has high productivity of oil and less emission of polluting waste during combustion, meeting the worldwide demand for sustainable energy sources. The aims of this research were the evaluation of response of the biologic activity measured by the optical technique of the biospeckle laser applied to macaw palm fruits at different maturity weeks and develop a classifier in function of biologic activity to determine the harvest period related with oil content in the fruits. To perform the experiment, 10 weeks fruits different maturity stages were evaluated. The biospeckle laser images were obtained by illuminating the epicarp of each fruit. The biological activity was quantified by absolute value of difference algorithm applied to biospeckle images. A neural network was developed to classify the fruits which were closer to harvest in function of biologic activity. Biologic activity showed a significant linear ratio (R^2 = 0.913) with the maturation of fruits. Classification results have shown that fruits from 59th week after flowering are ideal for harvest and present the highest oil levels.

Key words: Biologic activity, optical sensors, maturity, oil content.

INTRODUCTION

Brazil has stood out in the worldwide energy scenario, being considered to be one of the biggest producers and consumers of biodiesel. In spite of the advances made in this sector, the Brazilian energy matrix faces biodiesel production bottlenecks, due to the reduced amount of oilseed raw material. Currently, soybeans are the main source of biodiesel production, covering 74% of the raw material demand, corresponding to the production of 2.2 million m³ (ANP, 2014). This production is insufficient to sustain the demand for biodiesel projections in the

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coming years. The diversification of the raw materials used for the production of biodiesel is important for the continuous increase of the Brazilian bioenergy sector.

In this context, the macaw palm stands out because of the high production potential of oil from its fruits (Conceição et al., 2016), and the possibility to be used in a consortium with other species into agrosilvopastoral systems (Henderson et al., 1995; Viana et al., 2011). The estimate of oil productivity in the mesocarp is of 5000 $kg.ha^{-1}$ palm-kernel type, needed for biodiesel production, and of 1400 $kg.ha^{-1}$ of oil in the kernel is lauryl type, used in cosmetics (Clement et al., 2005; Barreto et al., 2016).

Despite the high productive potential of biodiesel, the palm tree presents some characteristics that hinder its exploitation in a large-scale production system (Motoike et al., 2013; Mota et al., 2011). Due to the variation in the maturity stage of the fruits, the period of harvest is still seen as a point that requires further study and development of specific technologies for the culture. The harvest realized with the fruits in different maturity stage reflects in oil quantity and quality (Hiane et al., 2005). Currently, the period to start macaw palm harvest is determined by the natural fall of fruits from the bunch. However, the contact of the fruit with the soil results in reduced oil quality due to an increase in oil sourness caused by microorganisms (Queiroz et al., 2015).

To determine physiologic maturity, direct methods of measuring are used, which take into account the physical and chemical features of fruit and vegetable (Kluge et al., 2010; Pinto et al., 2013). Despite the reliability, these analyzes generally present disadvantages such as sample deterioration, high analysis time and high costs (Chitarra and Chitarra, 2005; Santos et al., 2013).

Regarding the estimate on macaw palm oil content, one of the main analytic methods applied is the ASE (Accelerated Solvent Extraction) which uses a combination of temperature, pressure and solvent to extract the oil. However, this method is considered laborious, and requires the destruction of the fruit in the process, using up a great amount of the sample to perform analysis (Elfadl et al., 2010). The use of magnetic resonance significantly reduced the time spent during analysis; however, this method requires a prior preparation of the samples, which makes it difficult to use it (Panford and Deman, 1990).

Noninvasive measurement methods appear as an alternative to minimize these disadvantages. Optical instruments are being applied as an alternate to classify and control fruit and vegetable quality, with the use of spectral reflection data (Saeed et al., 2012; Viegas et al., 2016), the use of fluorescent sensors to determine maturity (Hazir et al., 2012), the use of digital images (Tan et al., 2010) and the use of biospeckle laser for quality and monitoring of biologic activity (Rabelo et al., 2005; Zdunek and Herppich, 2012).

Optical instruments have been used in the maturation analysis and oil content of the macaw palm. Matsimbe et al. (2015) used the spectroscopy of infrared and near infrared (VIS-NIR) applied to the macaw palm fruit mesocarp to estimate oil content. Although promising, these results still limit the measurement of the oil content without destroying the fruit, once the VIS-NIR models did not obtain significant results when applied directly in the epicarp.

The biospeckle laser technique has been successful as an alternative to traditional optical analysis for indirect measurement of biological material characteristics. Biospeckle images are formed when a biological material is illuminated by laser light, generating an interference pattern due to the scattering caused by structural cell tissue (Rabal and Braga, 2008). Observed through time, the interference patterns will be dynamic, once cell tissue activity result in a variation in the spread of the light reflected. This way, the intensity of the biospeckle pattern is associated to dynamic properties of the material analyzed. One of the main analysis methods of biospeckle is based on the quantity of dispersion of points around the diagonal of co-occurrence matrix by the calculation of the inertia moment (Arizaga et al., 1999) and the absolute value of difference (Cardoso and Braga, 2014). Biospeckle laser presents as an advantage the simple handling and low cost (Rabal and Braga, 2008; Zdunek et al., 2014).

The biospeckle laser technique has been applied to quantify and differentiate regions of different activities in several studies related to agriculture as in the analysis of leaf tissues (Ansari and Nirala, 2015), quality of meat (Amaral et al., 2015), root growth (Braga et al., 2009; Ribeiro et al., 2013), incidence analysis of parasites (Ansari et al., 2016; Grassi et al., 2016) and bacteria (Ramírez-Miquet et al., 2015).

In the analysis of fruit quality in specific, the biologic activity of fruit and vegetable obtained through biospeckle activity can be correlated with maturity (Chargot et al., 2012; Retheesh et al., 2016; Skic et al., 2016), senescence (Alves et al., 2013; Costa et al., 2017) and effect of storage temperature (Kurenda et al., 2012).

The objectives of this research were to analyze the response of the biologic activity measured by biospeckle laser technique applied to macaw palm fruits in different maturity weeks and develop a classifier in function of biologic activity, to determine the harvest period related with oil content in the fruits.

MATERIALS AND METHODS

Fruit collection

The fruits used in the experiment were collected in an area located in the Zona da Mata region, Minas Gerais state, located at 20º 23 '33" ' of latitude south and 47º 07 '31" 'of longitude west, with altitude of 601 m. The trees used were from a single population of the *Acrocomia aculeata* species with more than 10 years old, in reproductive state, cultivated into extractive system, with no commercial use and without prior soil preparation.

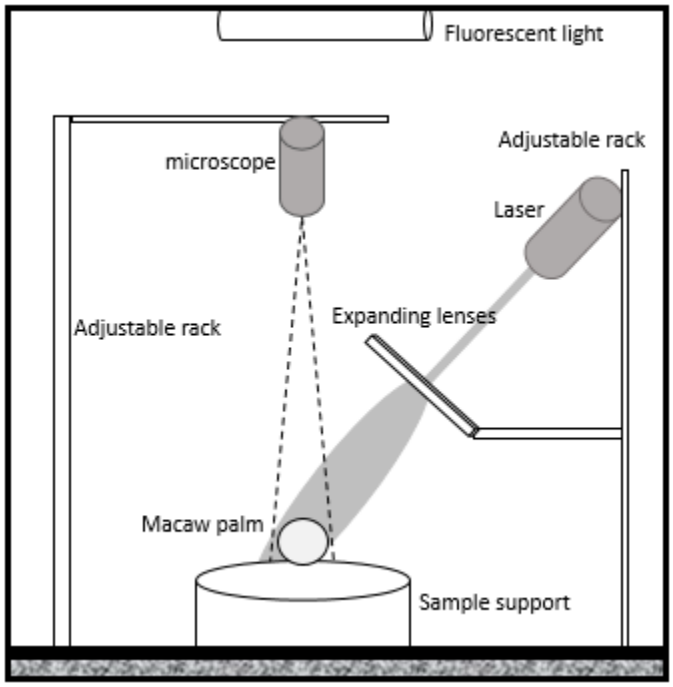

Figure 1. Experimental setting for image acquisition from biospeckle laser.

During the beginning of flowering, a bunch in each tree was marked randomly. After flowering 20 bunches from 20 different trees were selected. Ten fruit collections were performed between the 40th and the 61th week after flowering (WAF). In each collection, 100 fruits were acquired, being composed of 5 fruits of each bunch. The fruits were collected randomly disregarding fruit position within each bunch.

The collections were performed at intervals with progressive reduction, since maturity occurs more rapidly in the weeks approaching physiological maturation. Thus, between the first and the third collection there was a break of four weeks. Between the third and the eighth collection there was a break of two weeks. Finally, between the eighth and tenth collection there was a break of a week.

Analysis of fruit biologic activity using biospeckle laser technique

The analysis of the biologic activity obtained by biospeckle laser was performed in each fruit in all 10 weeks of maturity evaluated. The illuminations were made at the epicarp (peel) of each fruit, with no previous sample preparation and in an intermission of approximately 4 hours after fruit collection in the field.

To get images, an experimental unit was used, constituted by portable microscope (AM413ZT, 1280 x 1024 pixel resolution) for image acquisition connected straight to the USB port of a computer, a He-Ne laser (632 nm, 50 mW), movable rack and a set of lenses and intensity reduction filters. The laser beam was filtered with a neutral filter to reduce the intensity of light the beam was expanded using a converge lens to generate homogeneous illumination of samples. The field of view consisted of a window of radius of 2.5 cm. Laser has been positioned at 15 cm high from sample with an approximate inclination of 60° in relation to sample. The microscope was positioned 25 cm high from the position and with an approximate inclination of 20° in relation to sample and a 10x zoom. Figure 1 shows the disposition of the experimental settings of equipment in the experimental mobile unit.

The experimental setting that was used is backscattering, which records the light reflectance reflected by the fruit (Rabal and Braga, 2008). In each light session, for each fruits, 128 images (1280 x 1024 pixel resolution) were collected in 8 bits, relating to the biospeckle patterns in intermissions of 0.08 s. Activities measured in the experiment were allotted in frequency waves between 0 – 12.50 Hz. The software Speckle Tools (Godinho et al., 2012) was used to perform the image acquisition into biospeckle pattern collected by the portable microscope.

To quantify intensity variation from biospeckle patterns, the indexes of biologic activity were calculated in the fruit epicarp, using the algorithm of the absolute values of difference (Cardoso and Braga, 2014), according to Equation 1.

$$\mathrm{AB} = \sum_{ij}\left(\mathrm{M_{ij}}\frac{\mathrm{MOC\,ij}}{\sum_{\mathrm{ij}}\mathrm{MOC\,ij}}|i-j|\right) \quad (1)$$

Where Mij is the normal matrix value of co-occurrence in coordinates i and j, and MOCij is the real matrix value of co-occurrence in line i and column j.

An analysis of the colorimetric property hue was made with the purpose of demonstrating that the biological activity obtained in the different weeks of maturation reflect in quantitative colorimetric changes in fruits.

Determining oil content in fruits

Oil extraction was performed using n-hexane solvent into Soxhlet extractor by the method 032/IV from Analytical Norms from Adolfo Lutz Institute (IAL, 2005). Mesocarp was dried in a fanned heater at 65°C for 72 h. After drying, 5 g samples were placed in filter paper cartridges, and were arranged into the Soxhlet extractor immersed in 150 ml of n-hexane, for 8 h. In sequence, the extract was transferred to a heater at 105°C for 24 h for the evaporation of n-hexane and water contained in the oil. Finally, cooling to ambient temperature was performed followed by weighing. Oil extraction was performed for each fruit individually, which generated an oil content value for each evaluated sample (Equation 2).

$$\mathrm{OC} = \left(\frac{\mathrm{Mo}}{\mathrm{Ms}}\right)100 \quad (2)$$

Where OC stands for oil content in percent, Mo stands for oil mass in grams and Ms stands for sample total mass in grams.

RESULTS

The analysis of variation values for biologic activity was performed by boxplot, grouping five fruits in a bunch in each maturity week. Values that were out of inferior or superior limits were considered outliers and eliminated.

After elimination of outliers, the average biologic activity value was calculated for each of the 10 weeks of collection. From these values, a simple linear regression was applied to evaluate the relation between the number of weeks after flowering and biologic activity of fruits. A variation analysis was used to verify the significance of the regression model used at 5% significance level.

To evaluate biospeckle technical capacity in differing maturity weeks to determine the harvest period of fruits, a cluster analysis was carried out with a non-supervised

Table 1. Number of fruits constituted in each class.

Classification		Number of fruits per WAF	Total of fruits in each class
Immature Class (A)	41°WAF	94	181
	45°WAF	87	
Mature Class (B)	60°WAF	70	139
	61°WAF	69	

Where, WAF= weeks after flowering.

classifier which used k-means algorithms. The biologic activity averages in each harvest week were grouped into two classes, from the shortest distance to the center of each class. Ten interactions were performed, until no values has been incorporated into a different class that was in the former interaction.

It was determined that the biological activity values of the first class indicated that fruits were immature and not suitable for harvest. The biological activity values of the second class indicated that fruits were ripe and ready for harvest.

Correlation between oil content and biologic activity were analyzed during fruit maturity by Pearson coefficient at 1% significance.

Fruit classification to determine harvest moment in function of biologic activity

For the development of the classifier, two classes were defined according to the fruit maturity stage. The Immature class (A) was comprised of fruits collected in 41°WAF and 45°WAF. These fruits were chosen to represent the class of immature, for they were gathered in the first two collections, which are considered inappropriate for harvest. The mature class (B) was comprised of fruits gathered in 60°WAF and 61°WAF, that is, in the last two weeks of harvest, when the fruits were considered closer to physiologic maturity and therefore, good for harvest. In Table 1, it is presented the number of fruits for each class.

A feedforward backpropagation neural network was trained to classify the fruits in the immature and mature classes according to biologic activity measured by biospeckle laser, using Levenberg-Marquadt variable to accelerate training time and improve the performance during classification (Demuth et al., 2008).

Two architectures have been evaluated. The neural network architectures 1 (NN1) was comprised by an input layer, an intermediate layer with two neurons, an output layer with two neurons (Figure 2a). The neural network architectures 2 (NN2) was comprised by an input, two intermediate layers and one output layer with two neurons (Figure 2b). Biologic activity was used as an input descriptor. The intermediate and output layers used hyperbolic tangent as activation function. A binary system was used in the output layer, being 0 related to fruits classified into Immature class (A) and 1 refer to fruits classified into Mature class (B), considered able for harvest.

It was used 50% of the fruits to train, 20% for validation and 30% for tests the trained neural networks. Each structure was trained 10 times, since in the beginning of the training the network parameters are generated randomly and these values influence. Training was interrupted by the early stopping, when error increase in validation samples occurred in 6 consecutive cycles. The neural network architecture that presented each hit on the test classification was selected.

Performance in the chosen nets was determined through confusion matrix collected from the classification of the sample test (Congalton, 1991). Accuracy and comparison were determined by overall accuracy coefficient and Kappa index. Significance from Kappa indexes was analyzed through Z testing, which enabled to verify if classification in the neural networks was considered better than a random classification (De Leeuw et al., 2006).

The selected neural network was applied to fruits in the 10 maturity weeks evaluated, and the percentage of classified fruits pertaining to Immature class (A) and Matured class (B). The predominant classification of fruits taken from a bunch, determined the class for each bunch.

RESULTS AND DISCUSSION

Relation between biologic activity and fruit maturity

Figure 3 depicts a model for the response of biologic activity measured in the fruit epicarp during maturation. R^2 of 0.9127 for biologic activity adjustment in the epicarp shows that biospeckle laser has the capacity of being an efficient technique in pointing out maturity level, which can be applied directly to the peel of macaw palm fruit. Zdunek and Herppich (2014) used the biospeckle technique to predict in a non-destructive way the maturity stage for apples. Similar to the results observed for macaw palm maturity, an increase was observed in biologic activity during the period of fruit development, being more evident in periods of more advanced maturity.

When penetrating in plant tissues the laser light

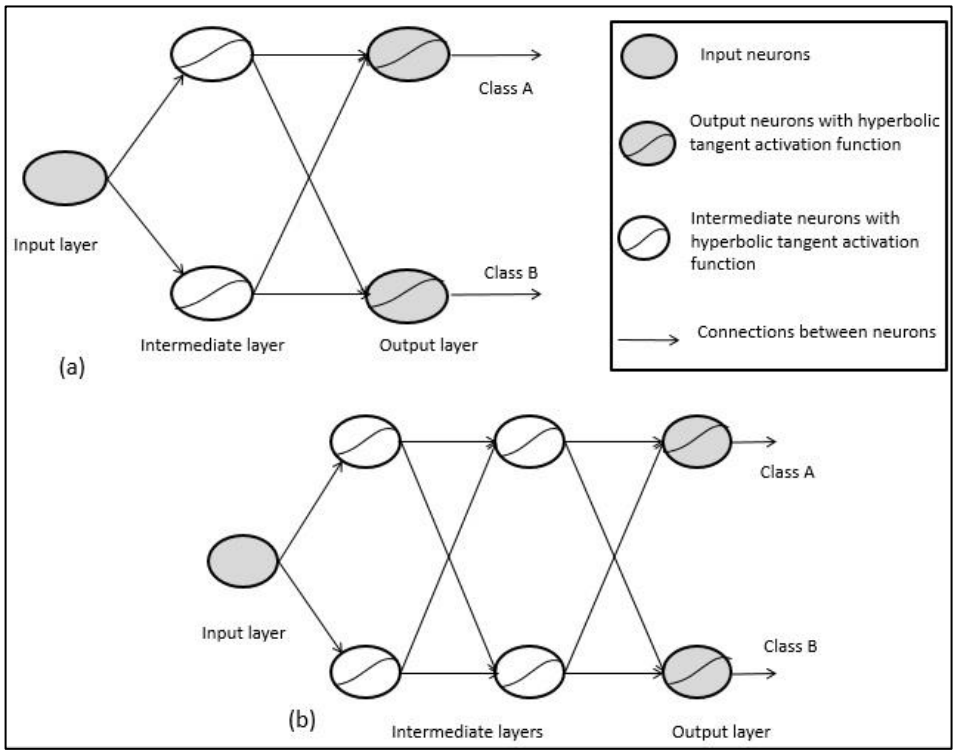

Figure 2. Representation of architectures Neural Network 1(a) and Neural Network 2(b).

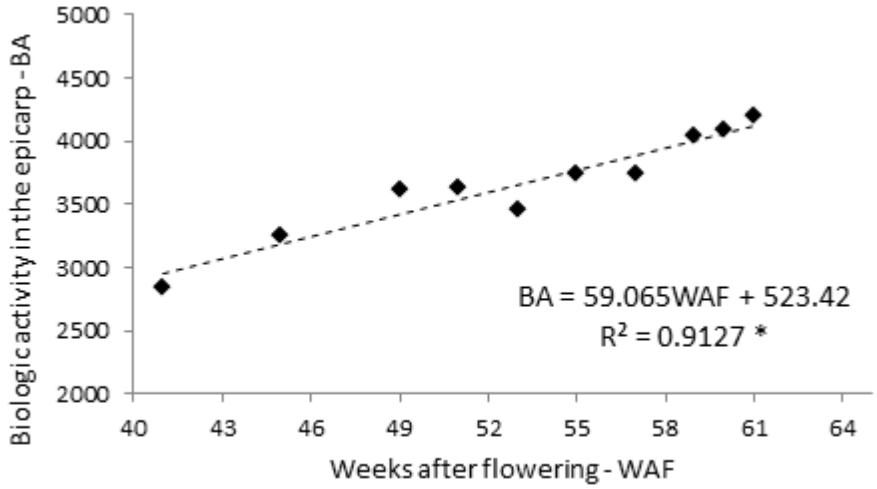

Figure 3. Analysis of simple linear regression related to biologic activity in the epicarp and number of weeks after flowering significance at level 5%.

undergoes scattering in different directions due to Cytoplasmic movement existing in the superficial and subsurface layers of tissues (Rabelo et al., 2005). The cells that compose the plant tissue act as scattering centers of the light beam, generating multiple refractions and reflections which produces the laser biospeckle patterns. In plant tissues the exchange of materials between organelles and and the transport of nutrients and enzymes happens near the cell walls, place where it occurs the interaction light and biological material. Changes in cellular characteristics result in alteration of the scattering centers of the light beam resulting in quantitative variation of the biological activity measured by biospeckle laser (Rabal and Braga, 2008).

During fruits maturation, cytoplasmic flows, breath, growth and cell division are factors responsible for

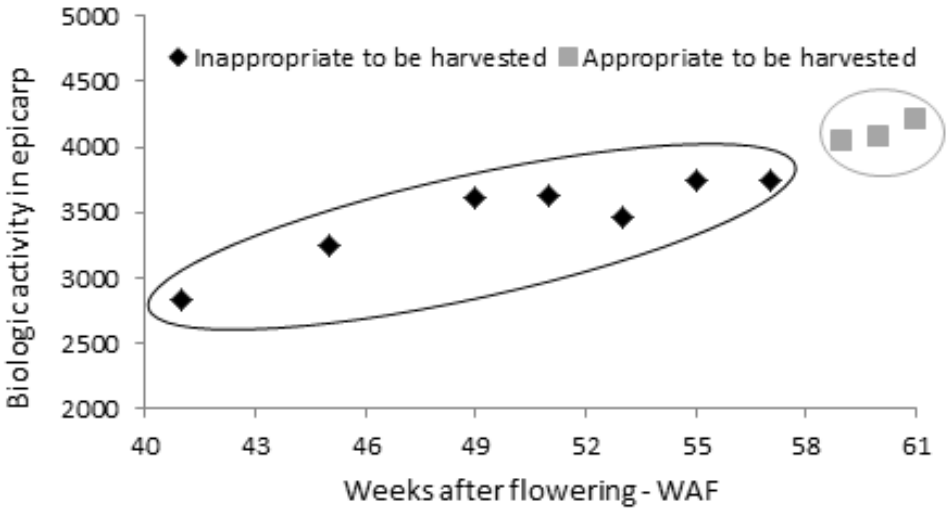

Figure 4. Grouping in classes (K-means) to distinguish fruits in moments considered inappropriate to be harvested and in moments considered appropriate to be harvested having biologic activity in the epicarp as a basis.

determine the activity of biospeckle (Bragra et al., 2009). Thus, quantified biological activity by biospeckle can be understood as an index related to the mobility and vitality of a tissue.

The complexity of plant tissues makes it difficult to understand the cause and effect related to biospeckle activity. Zdunek and Cybulska (2011), demonstrate that the degradation of the starch during ripening of apples affects the optical properties of the fruit tissues. According to the authors, the starch particles do not move along with the cytoplasmic flow, forming scattering centers of laser light. Thus, the more starch particles within the cells, higher the biospeckle activity.

Another aspect to be considered, is the absorption of laser light by plant tissue. In this case chlorophyll has an important role, since this type of material efficiently absorbs light in the visible spectrum (Rabal and Braga, 2008). Chlorophyll levels tend to decrease during maturation, decreasing the absorption of laser light and consequently increasing the scattering intensity and the values of biological activity. Nassif et al. (2012) and Hu et al. (2013) demonstrate a significant correlation between chlorophyll and biological activity for various fruits. In the case of macaw palm, more in-depth studies are necessary with the objective of obtaining a relation between the biological activity and physiological factors during a maturation.

When analyzing the grouping into classes (Figure 4) it was possible to observe the distinction of fruits considered immature, and thus, inappropriate for harvest, and fruits considered mature and ready for harvest having biologic activity in the epicarp in the 59th WAF, showing that from this point on fruits are ready to be harvested.

The analysis of the colorimetric property hue reinforce the results obtained by biospeckle laser technique demonstrated that the biological activity reflects quantitatively the physiological changes of the fruits in the different weeks (Figure 5). Fruits near the harvest period tended to lower values of the hue when compared to fruits in lesser degree of maturation. The fruits between 41st and 49th WAF had a concentration in the value of the hue close to 60°, indicating a medium tonality close to yellow. The fruits from the 59th WAF presented a hue with an average value of 45°, indicating a yellowish red tonality.

Analysis of oil content throughout maturity weeks

The closer it gets to fruit physiologic maturity, the higher were oil contents found in bunches (Figure 6). In advanced maturity stages, it was noticed a variation in oil content between 47 and 66% among the observed bunches. Bunches b1, b10, b13, b16 and b18 presented outliers in at least one of the maturity stages.

The correlation of 0.959 (significant at level 0.01) between the biological activity and the oil content showed that biologic activity could also be used as a parameter indicator of oil content in the fruits, which allows for the generation of a relationship between both parameters.

The synthesis and accumulation of oil in the fruits are directly connected to availability of starch reserves in the form of sugars (Chitarra and Chitarra, 2005). Montoya et al. (2016), demonstrated that in the fruits of macaw palm the reduction of starch content coincides with the increase in oil content. As highlighted, the particles generated by the degradation of the starch in the formation of sugars form laser light beam scattering centers, which increases the biological activity measured

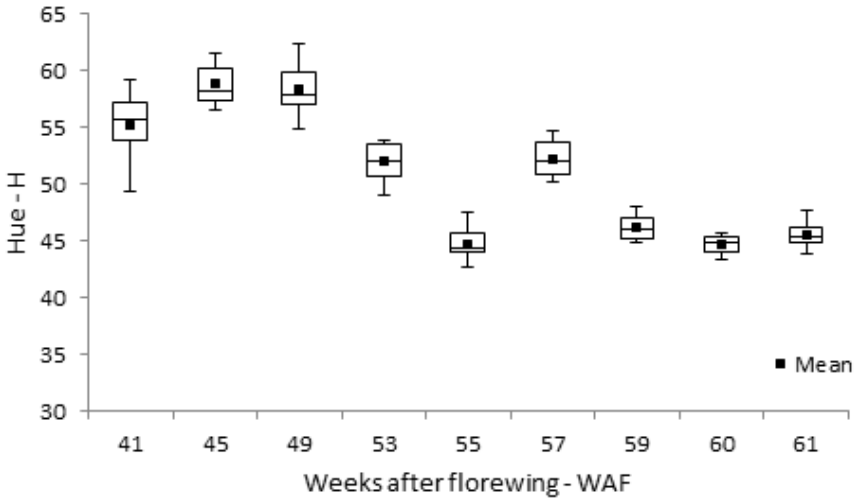

Figure 5. Analysis of the colorimetric property hue in the different weeks of maturation of macaw palm.

Table 2. Parameters for accuracy verification in the net used in the test group.

Parameter	NN 1	NN 2
Global Efficiency (GE%)	80.21	82.29
Kappa Coefficient (K)	0.60	0.65
Zc	7.2922*	8.8047*

*Significance at level 0.01.

by laser biospeckle. In this way, the hypothesis is suggested that the degradation of the starch during maturation of fruits macaw palm can be one the factors responsible for the correlation between oil content and biological activity.

Fruit classification in the moment of harvest related to biologic activity

Two classes were established according to the maturation stage of the fruit. Immature class was comprises of fruits gathered in 41°WAF and 45°WAF and considered inappropriate for harvest. The Mature class was comprised of fruits in 60ºWAF and 61°WAF, period considered close to the ideal moment to be harvested.

The Kappa coefficient showed that classifications performed by both neural networks were better than a random classification at significance level 0.01 (Table 2). NN2 presented a higher global efficiency and it was chosen as the most appropriate net where fruit classification can be performed.

When analyzing fruit classification in the 10 maturity weeks, it has been observed that fruits harvested until 57°WAF were predominantly classified into immature class, presenting average oil content of up to 45.50% (Table 3). The percentage of classification of harvested fruit in the 55th WAF and 57th WAF (close to 50.00%), showed that these dates the number of immature fruits to be harvested and fruits ready to be harvested are close by, making classification variable. From the 59th WAF fruits were classified predominantly into mature class, being considered ready to be harvested, when biologic activity was used as an evaluation parameter for the maturity stage. In these stages, the fruits show the average oil content higher (above 45.96%).

In extreme maturity stages, in 41th WAF and 61th WAF, the lowest errors in classification were detected, 17.02 and 11.59%. In these cases, classification error can be associated to experimental imprecisions during the application of biospeckle technique. The presence of damage in the lightened region of the epicarp such as micro chinks, cracks and darken spots May also have influenced the biological activity and chlorophyll levels in the fruit epicarp.

The period between the 55th WAF and 57th WAF presented the smallest difference between the percentage of classified fruits in the Immature and Mature classes, showing that in stages close to harvest, the classification by biologic activity has its accuracy reduced. The non-uniform maturity, mainly in fruits coming from different bunches, might have reflected in

Table 3. Fruits classified as Immature (A) and Mature (B) though neural network NN2.

Activity	NF	Immature Class %	Mature Class %	OC (%)	PC
41 WAF	94	82.97	17.02	9.68	A
45 WAF	87	72. 41	27.59	22.56	A
49 WAF	90	58.89	41.11	31.02	A
51 WAF	95	57.95	42.05	32.63	A
53 WAF	71	71.84	28.16	39.58	A
55 WAF	67	50.74	49.25	44.68	A
57 WAF	88	56.82	43.18	45.50	A
59 WAF	78	20.51	79.49	45.96	B
60 WAF	70	20.00	80.00	50.67	B
61 WAF	69	11.59	88.41	53.02	B

Where: NF = number of fruits; OC (%) = Oil content; PC = predominant class.

Table 4. Predominant Classification (PC) for each bunch through neural network NN2 in the analyzed maturity weeks. Immature Class (A) and Mature (B).

Classification	WAF (Week After Flowering)									
	41	45	49	51	53	55	57	59	60	61
Bunch 1	A	A	A	B	B	A	A	A	--	--
Bunch 2	A	A	A	A	A	A	B	B	B	B
Bunch 3	A	B	B	A	--	A	A	B	B	B
Bunch 4	A	A	A	A	A	A	B	--	--	--
Bunch 5	A	A	A	A	A	--	B	B	B	B
Bunch 6	A	A	B	A	A	B	B	B	--	--
Bunch 7	B	A	B	B	B	A	A	B	B	B
Bunch 8	B	A	A	A	A	A	A	B	B	B
Bunch 9	A	A	A	B	A	--	A	A	B	B
Bunch 10	A	B	B	B	A	--	A	B	B	B
Bunch 11	A	A	A	A	A	B	B	B	--	--
Bunch 12	A	A	B	B	A	A	A	B	B	B
Bunch 13	A	A	B	B	B	--	B	B	B	B
Bunch 14	A	A	A	B	A	B	A	B	--	--
Bunch 15	A	A	A	A	--	B	B	B	B	B
Bunch 16	A	A	A	A	A	--	A	B	B	B
Bunch 17	A	B	A	B	A	B	A	B	B	B
Bunch 18	A	A	A	A	A	A	B	B	B	B
Bunch 19	A	A	A	A	A	--	A	A	B	B
Bunch 20	A	A	A	B	A	A	A	B	B	B
PC	A	A	A	A	A	A	A	B	B	B

the variant of biologic activity in this period.

It is important to point out that although anticipated harvest allows for loss reduction of the mature fruits through natural fall and of the number of times in the performance of field harvest compared to selective harvest (Skic et al., 2016), it will demand a careful handling in the post-harvest phase so that the fruit reach the highest levels of quality and quantity of oil content.

In specific situations where the classification of each fruits becomes unfeasible, a general evaluation of the bunch might be an option for analysis in the harvest time. In Table 4 the general classification per bunch in each maturity week is depicted. Bunches were classified predominantly in the immature class, being considered inappropriate to be harvested up to the 57th WAF. From the 59th WAF the bunches were predominantly classified in the Mature class, it is a period they are considered ready by the classificator to perform macaw palm fruit

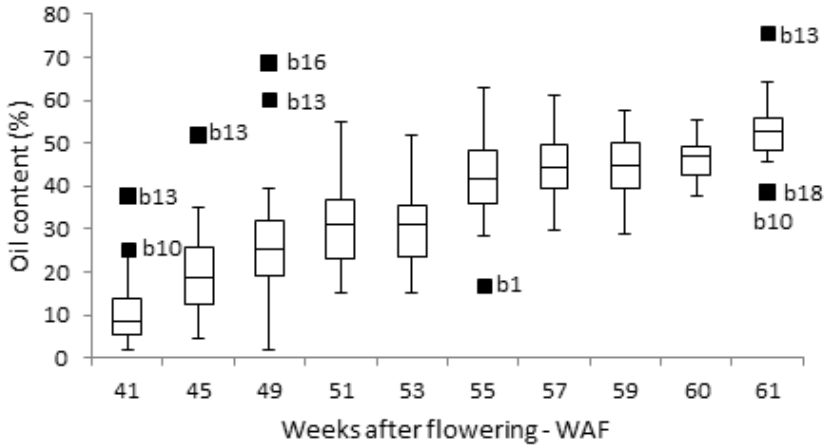

Figure 6. Variation in oil content in bunches in analyzed maturity stages.

harvest, with higher oil levels, when biologic activity is used as a parameter to evaluate maturity stage.

Based on the results, biologic activity measured through biospeckle laser can be considered a viable parameter for maturity evaluation and a promising tool when it comes to indicating the harvest period for macaw palm fruit. Sensors built from biospeckle laser technique might be used in automated selection systems to evaluate fruit conditions in relation to its oil content and to verify bunch condition at harvest directly in field.

Conclusions

An increase in biologic activity was observed throughout maturity weeks after flowering for macaw palm fruits when the epicarp was analyzed, presenting a significant linear relation between biologic activity and maturity weeks. These results showed the effectiveness of optical capacity from biospeckle laser technique in following the development of maturity for macaw palm fruits.

Oil level obtained from macaw palm bunches presented an increase throughout fruit maturity, whose higher oil levels were found closer to physiologic maturity of fruits. The developed classifier showed that the 59th week after flowering was considered the ideal period for harvest, when biologic activity was used as a parameter to set maturity, showing the applicability of biospeckle laser to determine the harvest period for macaw palm fruits.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Postharvest diseases of tomato and natural products for disease management

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Gray mold and soft rot are the most important postharvest diseases of tomato worldwide. A survey of fresh-market tomato fruit was conducted in Oahu to determine which fungal and bacterial pathogens were most commonly associated with postharvest disease. *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Mucor*, *Stemphyllium*, *Rhizopus* and *Penicillium* were the most frequently isolated fungi and *Acetobacter*, *Gluconobacter*, *Klebsiella*, *Leuconostoc* and *Pectobacterium* were the prevalent bacteria. Fifty-one percent of the diseased tomatoes had been imported from California and Mexico and 49% had been grown locally at three sites in Oahu. Pathogenicity tests revealed that 33 of 99 fungal isolates and 10 of 17 bacterial isolates were pathogenic on tomato types known as common market, cherry and grape tomato. Based on fruit assays, *Botrytis cinerea* (B03) and *Pectobacterium carotovorum* (BA17) were the most virulent isolates. Tested leaf extracts of *Capsicum annuum* cv. Stocky Red, *C. annuum* cv. Criolla de cocina, *Capsicum chinense* cv. NuMexsuave, *Tagetes tenuifolia*, *Aloe vera*, *Origanum vulgare* and *Azadirachta indica* were ineffective as biopesticides and did not reduce spore germination or mycelial growth of *B. cinerea* (B03) nor *P. carotovorum* (BA17). In contrast, a proprietary product (PF) reduced mycelial growth of *B. cinerea* (B03) and was further evaluated at doubling concentrations ranging from 0.0625 to 1 ml/L. Mycelial growth of *B. cinerea* and other fungi was completely inhibited by exposure to PF at 1 ml/L. On the other hand, PF was not an effective biopesticides against *P. carotovorum*. PF shows promise for reducing gray mold and will be evaluated as a preharvest spray on tomato plants in the greenhouse.

Key words: Survey, postharvest diseases, tomato, natural product, *Botrytis cinerea*, *Pectobacterium carotovorum.*

INTRODUCTION

Public concern about fungicide residues on raw fruits and vegetables has stimulated research efforts using natural products to reduce incidence of postharvest diseases. Approximately, 25 and 38% of harvested fruits and vegetables, respectively, are lost to postharvest spoilage in the U.S. and global markets (Kantor et al., 1997). Fresh fruit and vegetables can be infected by pathogenic fungi and bacteria during crop growth in the field,

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harvesting, postharvest, storage and consumption (Barth et al., 2009). Postharvest diseases cause economic losses in field because of added costs of harvesting, transportation and storage (Adikaram, 1986). The current study focuses on tomato (*Solanum lycopersicum* Mill), which is one of the most important vegetables produced globally, comprising approximately 14% of world vegetable production (Kader, 2004; FAO, 2003). The production value of tomato, estimated at more than $50 billion, makes it the fourth most important commercial crop in the world (Vincent et al., 2013). Tomato is also one of the leading vegetable crops in Oahu, Hawaii. The first objective of this work was to determine the most serious postharvest diseases of tomato in Oahu and to identify the most virulent pathogens associated with each disease. The second objective was to evaluate natural products for biopesticidal activity against pathogenic fungi and bacteria associated with tomato postharvest diseases.

MATERIALS AND METHODS

Survey of postharvest diseases in tomato fruit

A survey was conducted in Oahu, Hawaii extending from November 2014 to April 2015. Samples of infected tomato were randomly collected from 17 locations and 37 markets in Oahu. Two samples (each sample consisting of 10 fruits) were selected from each market. Tissues showing symptoms of postharvest disease were cultured to identify associated pathogens. The percentage of infected tomato based on origin (local or imported) was reported.

Isolation and identification of pathogens

Small (1 cm) sections of infected fruit were cut and surface-sterilized individually in 2% sodium hypochlorite for 1 min and rinsed twice in sterile distilled water. The pieces were dried between sterile Whatman No.1 filter paper and cultured on water agar plates and incubated at 28±2°C for 24 h. Single hyphal tips were transferred to Petri dishes containing V8 medium and incubated at 28±2°C for 5 days under a 12 h photoperiod (Carisse and Van Der Heyden, 2015). Purified cultures were visually identified utilizing laboratory manuals (Dugan, 2006). For bacterial isolates, small sections of rotted tissues were suspended in distilled water for two minutes and the suspension was streaked onto the surface of nutrient agar (NA) plate and plates were incubated at 30°C for 24 h. Basic bacteriological tests including KOH sensitivity, oxidation/fermentation (OF), production of catalase, degradation of sodium polypectate, and hydrolysis of esculin and starch were conducted on each isolated bacteria. All bacterial strains were maintained in freezers (-80°C) until used. Presumptive identifications were confirmed with 16S rDNA sequence analysis (Weisburg et al., 1991).

Pathogenicity tests

Pathogenicity tests were performed on all fungal and bacterial isolates on three types of tomato fruits as previously described by Ahmed et al. (2016). Fruits were selected to be uniform in size and color, free from wounds and showing no symptoms of disease. Fruit were washed with tap water, surface sterilized by dipping in 1% sodium hypochlorite solution for 10 min, rinsed by dipping twice in sterile distilled water for at least 10 min, and dried in ambient air. A wound (1 mm diameter in 4 mm deep) was made on each fruit using a pipette tip. Mycelial plugs from 10-day-old-cultures of the fungal isolates were inserted into wounds using 0.2 to 10 µl pipette tips. For bacterial stains, fruit were inoculated with 20 µl of a bacterial suspension ($1x10^8$/CFU). Inoculated fruit were placed in plastic box containing sterile paper towels moistened with sterile water and incubated for 72 h at 23°C. An organism was recorded as pathogenic if symptoms of rot appeared on the tested fruit. The experiments were set up separately for fungal and bacterial isolates with four replications and each experiment was repeated twice.

Virulence tests

Tests were conducted to determine the most virulent isolates on each of the three types of tomato fruit (common market, cherry and grape). Fruits selected were uniform in size and color, free from wounds and showing no symptoms of disease. Virulence of each isolate was determined by measuring the lesion diameter of inoculated fruit after incubation at 23°C for 72 h. The experiments were set up as complete randomized design (CRD) with four replicates. Data were analyzed using SAS 9.2 V.USA and means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. The tests were repeated twice.

Molecular identification

Fungal DNA was extracted from freshly collected mycelium of 10-day-old cultures using the Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc.). The ITS region of the fungal isolates was amplified with the primer pair ITS3 (5-GCA TCG ATG AAG AAC GCA GC-3) and ITS4 (5- TCC TCC GCT TAT TGA TAT GC-3) (Nikolcheva et al., 2003).

Bacterial DNA was extracted from overnight cultures using the Microbial DNA Isolation Kit (MO BIO, Laboratories, Inc.) according to manufacturer's instructions. The 16S rRNA was amplified by PCR for all the isolates using the primers: 16S forward primer (5'-AGAGTTTGATCCTGGCTCAG-3) and 16S reverse primer (5′ACGGCTACCTTGTTACGACTT-3′). PCR was performed as previously described by Srinivasa et al. (2012) and Weisburg et al. (1991). Each PCR reaction was run with a negative control (no DNA). The PCR products were electrophoresed on 1.5% agarose gels, stained with 0.4 µg/ml ethidium bromide, and bands visualized with a UV illuminator.

Sequence analysis

Sequence analysis was conducted as described in previous work (Ahmed et al., 2016). PCR product was cleaned utilizing ExoSAP-1T (Affymetrix, Inc., USA). The 5 µl of post-PCR reaction and 2 µl ExoSAP-IT reagents were mixed. The mix was incubated at 37°C for 15 min followed by incubation at 80°C for 15 min. Each purified template was sequenced on both strands using two flanking primers (ITS3- ITS4) for fungal isolates and 16s primers for bacteria. The sequences of ITS 3 and 4 regions, 16s of the tested isolates were edited in order to generate a consensus sequence from forward and reverse sequence in the amplicon using sequence assembly software (DNA BASER). A consensus sequence was analyzed by NCBI BLAST database for fungal and bacterial identities.

Natural controls

Leaf extracts made from *Capsicum annuum* cv. Stocky Red,

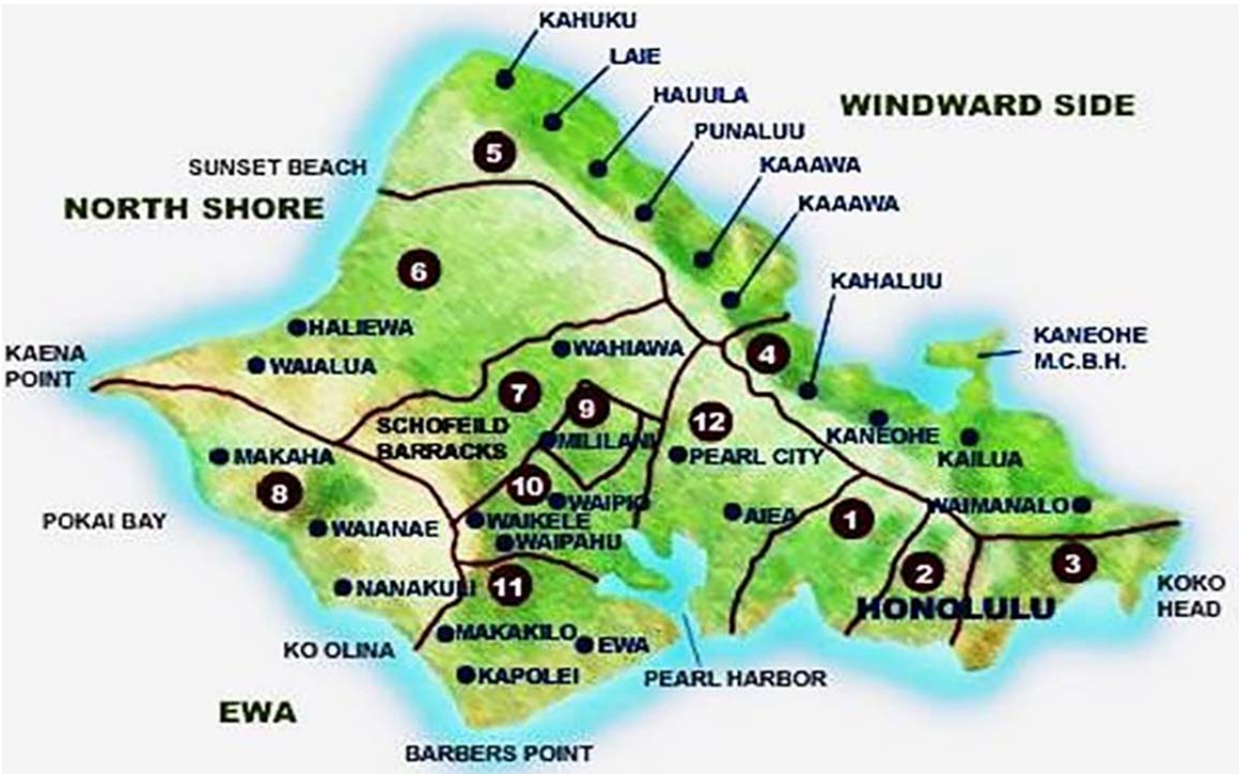

Figure 1. Map of Oahu showing sites where fruit samples were collected. Numbers refer to geographic divisions in the island of Oahu. Dots show sites where fruits were collected from separate markets. http://katebraden.com/neighborhoods.shtml.

Capsicum annuum cv. Criolla de cocina, *Capsicum chinense* cv. NuMexsuave, *Tagetes tenuifolia*, *Aloe vera* (leaves and gel), *Origanum vulgare* and *Azadirachta indica* (Neem oil) and a proprietary formulation (PF) (Agrichem, Inc., Australia) were tested. Leaves were extracted by following method described by Wilson et al. (1997) with some modification. Raw leaves were collected in plastic bags and freeze for a minimum of 12 h at 20°C. Plastic bag was removed and leaves fluid were sterilized by using 0.22 µm Millipore filter and stored in 4°C until used.

Assays of antifungal activity

The antifungal activity of nine plant extracts was evaluated against *Alternaria* and *Botrytis* isolates by using an inhibition assay described as the 'poisoned food method' (McCutcheon et al., 1994) with slight modification. A 5 ml sterilized crude extract was mixed with 15 ml of 45°C cooled molten V8 medium and allowed to solidify at room temperature for 30 min. A mycelial disc 6 mm diameter of 7 to 10-day-old cultures was transferred to a Petri plate containing V8 and crude extract. The V8 plate without plant extract served as a control. The antifungal activity of PF was evaluated against *B. cinerea* (B03) at five concentrations 1, 0.5, 0.25, 0.125, 0.0652 ml/L. The most effective PF concentration was reevaluated for the remaining 33 pathogenic fungal isolates. Since different genera have different growth rates on V8 medium, a separate control was established for each fungus by recording the time needed for mycelium to reach the edge of the Petri dish. At that point, the corresponding plate containing PF was measured for inhibition. The experiments were conducted as complete randomized design (CRD) with four replications. Data were analyzed using SAS 9.2 V.USA. Means were compared by Duncan's multiple range tests. Differences at $p < 0.05$ were considered significant. Each experiment was repeated three times.

RESULTS AND DISCUSSION

Survey

Ninety nine fungal and seventeen bacterial isolates were recovered from tomato from the 37 markets in Oahu (Figure 1). Fungal genera were *Alternaria*, *Botrytis*, *Colletotrichum*, *Fusarium*, *Geotrichum*, *Mucor*, *Stemphylium*, *Rhizopus* and *Penicillium*. Bacterial genera were *Acetobacter, Gluconobacter, Klebsiella*, *Leuconostoc* and *Pectobacterium* (Figure 2 and Table 1). Examination of diseased tomatoes based on origin showed 51% were imported from California and Mexico and 49% were grown locally at three sites in Oahu. Some of these pathogens are known to survive on fruit and be spread during transportation, handling and storage (Barkai-Golan, 2001).

Pathogenicity and virulence

The pathogenicity tests showed that 33 of 99 fungal

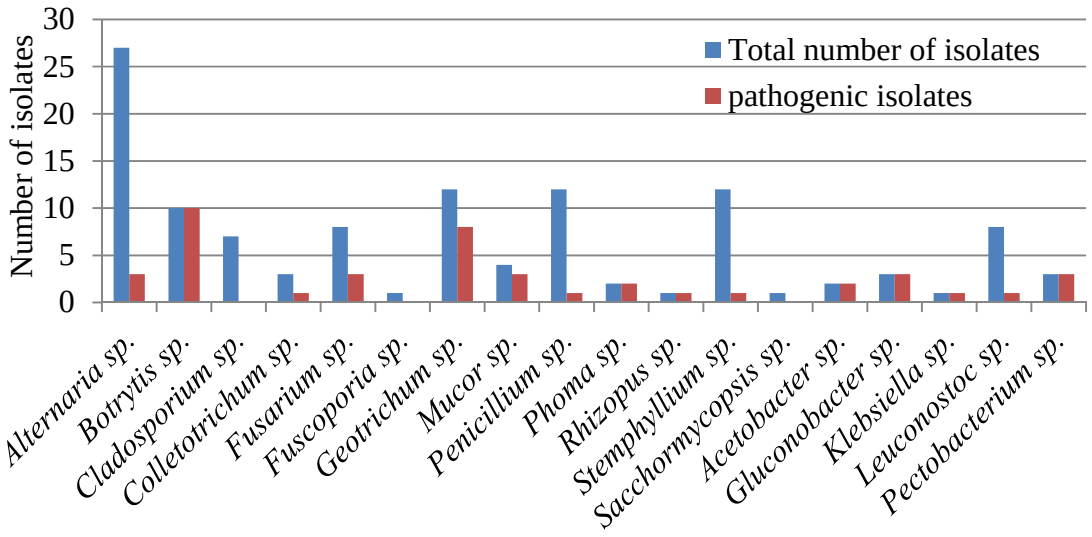

Figure 2. Genera of fungi and bacteria isolated from infected tomato fruit during a market survey in Oahu.

Table 1. Characteristics of bacterial isolates obtained from infected tomato fruit.

Bacterial stains	Strain No.	Gram[a]	O/F	Pectolytic enzyme production	Esculin hydrolysis	Starch hydrolysis	Catalase production
Gluconobacter frateurii	BA01	-	+/+	-	-	-	+
Klebsiella oxytoca	BA02	-	+/+	-	+	-	+
Leuconostoc sp.	BA03	+	+/-	-	+	-	+
Leuconostoc sp.	BA04	+	+/-	-	+	-	+
Acetobacter sp.	BA05	-	+/+	-	-	-	-
Leuconostoc sp.	BA06	+	+/+	-	-	-	+
Leuconostoc sp.	BA07	+	+/+	-	-	-	+
Leuconostoc sp.	BA08	+	+/-	-	-	-	-
Gluconobacter frateurii	BA09	-	+/+	-	-	-	+
Leuconostoc sp.	BA10	+	+/+	-	+	-	+
Leuconostoc sp.	BA11	+	+/+	-	+	-	+
Pectobacterium carotovorum	BA12	-	+/+	+	+	-	+
Acetobacter sp.	BA13	-	+/+	-	-	-	+
Gluconobacter frateurii	BA14	-	+/+	-	-	-	+
Pectobacterium carotovorum	BA15	-	+/+	+	+	-	+
Leuconostoc citreum	BA16	+	+/+	-	-	-	-
Pectobacterium carotovorum	BA17	-	+/+	+	+	-	+

[a]Presumptive Gram stain determined by formation of sticky stands 30s after adding KOH which is indication of a Gram negative for bacterial cells.

isolates and 10 of 17 bacterial isolates were pathogenic on all three types of tomato fruit (Table 2). Other reports indicate that fungi and bacteria survive and grow saprophytically on tomato (Smilanick, 2004; Agrios, 2005). In this study, the fungal isolates varied in virulence. The pathogenic isolates of *B. cinerea* were highest in lesion diameter range with 16-70, 10-32 and 10-25 mm on three types of tomato fruit common market, cherry and grape, respectively (Table 3). In addition, *B. cinerea* isolates were varied in their standard deviations, indicating that *Botrytis* isolates are different in virulence level. On the other hand, *B. cinerea* (B03) and *P. carotovorum* (BA17) were significantly more virulent than other isolates when tested on common market, cherry and grape tomato (Tables 4 and 5). Differences in virulence among pathogens are frequently a result of the

Table 2. Pathogenicity tests of fungal and bacterial isolates on artificially wounded fruit of three types of tomato 72 h after incubation at 23°C.

Isolates	Isolate no.	Types of tomato		
		Common market	Cherry	Grape
Alternaria sp.	A01- A03, A05- A19, A21	-	-	-
Alternaria sp.	A04, A20, A22	+	+	+
Botrytis cinerea	B01- B10	+	+	+
Colletotrichum sp.	C01	+	+	+
Colletotrichum sp.	C02	-	-	-
Fusarium sp.	F01- F02, F04, F06	-	-	-
Fusarium sp.	F03, F05,F07- F08	+	+	+
Geotrichum sp.	G01- G03, G05- G11	+	+	+
Geotrichum sp.	G04, G06	-	-	-
Mucor sp.	M01- M02	-	-	-
Mucor sp.	M03,M04	+	+	+
Penicillium sp.	P07	+	+	+
Penicillium sp.	P01-P06	-	-	-
Rhizopus sp.	R01- R03	+	+	+
Stemphylium sp.	S01- S02	+	+	+
Acetobacter sp.	BA5, BA13	+	+	+
Gluconobacter sp.	BA1, BA9, BA14	+	+	+
Klebsiella sp.	BA2	+	+	+
Leuconostoc *sp.*	BA3- BA4, BA6- BA8, BA10- BA11	-	-	-
Leuconostoc *sp.*	BA16	+	+	+
Pectobacterium sp.	BA12, BA15, BA17	+	+	+

+ = rotting; - = no rotting

Table 3. The average, range and standard deviation of virulence fungal isolates on common market, cherry and grape tomato 72h after incubation at 23°C.

Fungal genera	Isolates no.	Common market			Cherry			Grape		
		□	R	SD	□	R	SD	□	R	SD
Alternaria sp.	3	29	28-30	1.15	17	13-20	3.49	12	12-13	0.50
Botrytis cinerea	10	48	16-70	19.53	20	10-32	7.03	16	10-25	5.43
Colletotrichum sp.	1	33	-	-	19	-	-	15	-	-
Fusarium sp.	3	35	32-38	2.84	21	20-21	0.76	18	18	0.41
Geotrichum sp.	8	39	35-40	1.87	20	18-25	2.11	20	20	3.29
Mucor sp.	3	56	48-60	6.92	21	20-22	1.07	20	20	0.44
Penicillium sp.	1	35	-	-	21	-	-	12	-	-
Phoma sp.	2	35	31-40	6.12	17	17-17	0.00	11	11-11	0.00
Rhizopus sp.	1	60	-	-	27	-	-	20	-	-
Stemphylium sp.	1	30	-	-	19	-	-	13	-	-

differences in production of cell wall degrading enzymes (CWDEs), oxalic acid and/or secretion of pathogenicity factors (Bellincampi et al., 2014; Kubicek et al., 2014). All *Botrytis* and *Pectobacterium* isolates were pathogenic to the original host from which they were isolated (Figure 2). *B. cinerea* (B03) and *P. carotovorum* (BA17) produced significantly larger lesion diameters (Tables 4 and 5). In previous studies, *Botrytis* and *Pectobacterium* were some of the most important pathogens causing spoilage decay on tomato (Ahmed et al., 2016; Akbar et al., 2013; Etebu et al., 2013; Fillinger and Elad, 2015).

Molecular identification

A PCR product of 370 bp was amplified efficiently for all

Table 4. Virulence of *Botrytis cinerea* isolates on common market, cherry and grape tomato 72 h after incubation at 23°C.

isolates no.	Lesion diameter (mm)		
	Common market	Cherry	Grape
B01	68.00[a]	27.00[b]	20.00[b]
B02	30.00[c]	22.00[c]	10.00[c]
B03	70.00[a]	32.00[a]	25.00[a]
B04	30.00[c]	12.00[de]	12.00[c]
B05	30.00[c]	16.00[d]	10.00[c]
B06	16.00[d]	14.00[d]	13.00[c]
B07	59.00[b]	22.00[c]	18.00[b]
B08	60.00[b]	22.00[c]	20.00[b]
B09	61.00[b]	25.00b[c]	20.00[b]
B10	59.00[b]	10.00[e]	10.00[c]
Distilled water	01.00[e]	01.00[f]	01.00[d]

*Mean values followed by different letters within a column are different according to Duncan's multiple range tests ($p \leq 0.05$).

Table 5. Virulence of bacterial isolates in common market, cherry and grape tomato 72 h after incubation at 23°C.

Bacterial strains	Strain no.	Lesion diameter		
		Common market	Cherry	Grape
Acetobacter sp.	BA05	27.00[de]	20.00[d]	15.00[c]
Acetobacter sp.	BA13	35.00[c]	23.00[c]	17.00[b]
Gluconobacter sp.	BA01	30.00[d]	21.00[d]	15.00[c]
Gluconobacter sp.	BA09	24.00[e]	14.00[e]	10.00[d]
Gluconobacter sp.	BA14	30.00[d]	14.00[e]	11.00[d]
Klebsiella sp.	BA02	26.00[e]	12.00[f]	11.00[d]
Leuconostoc *sp.*	BA16	37.00[c]	23.00[c]	17.00[b]
Pectobacterium sp.	BA12	47.00[b]	29.00[b]	24.00[a]
Pectobacterium sp.	BA15	35.00[c]	23.00[c]	17.00[b]
Pectobacterium sp.	BA17	66.00[a]	31.00[a]	24.00[a]
Distilled water	control	01.00[f]	01.00[g]	01.00[e]

*Mean values followed by different letters within a column are significantly different according to Duncan's multiple range tests ($p \leq 0.05$).

fungal isolates. The ITS3-ITS4 region of identified fungi at > 98% similarity was compared with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *B. cinerea* was counterpart at 100% similarity. A PCR product with expected size 1400 bp was amplified for all bacterial isolates and NCBI BLAST of the 16S region identified bacteria at 99 to 100%. *Pectobacterium* isolates matched 100% with *P. carotovorum*.

Inhibitory effect of plant extracts on fungal colony

The crude leaf extracts of *C. annuum* cv. 'StockyRed', *C. annuum* cv. 'Criolla de cocina', *C. chinense* cv. 'NuMexsuave', *T. tenuifolia*, *A. vera*, *O. vulgare* and *A. indica* Neem oil showed no measurable inhibition of mycelial growth for *Alternaria* sp. or *Botrytis* sp. In contrast, PF completely inhibited mycelial growth of both fungi (Figure 3). The most effective PF concentration was 1 mL/L that completely inhibited growth of all 33 of the other tested pathogenic fungi (Table 6).

Conclusion

B. cinerea and *P. carotovorum* were the most virulent postharvest pathogens of tomato in Oahu. Thirty percent of the fungal and 58% of the bacterial isolates were pathogenic. A natural proprietary product (PF) had sufficient antifungal activity to completely inhibit mycelial

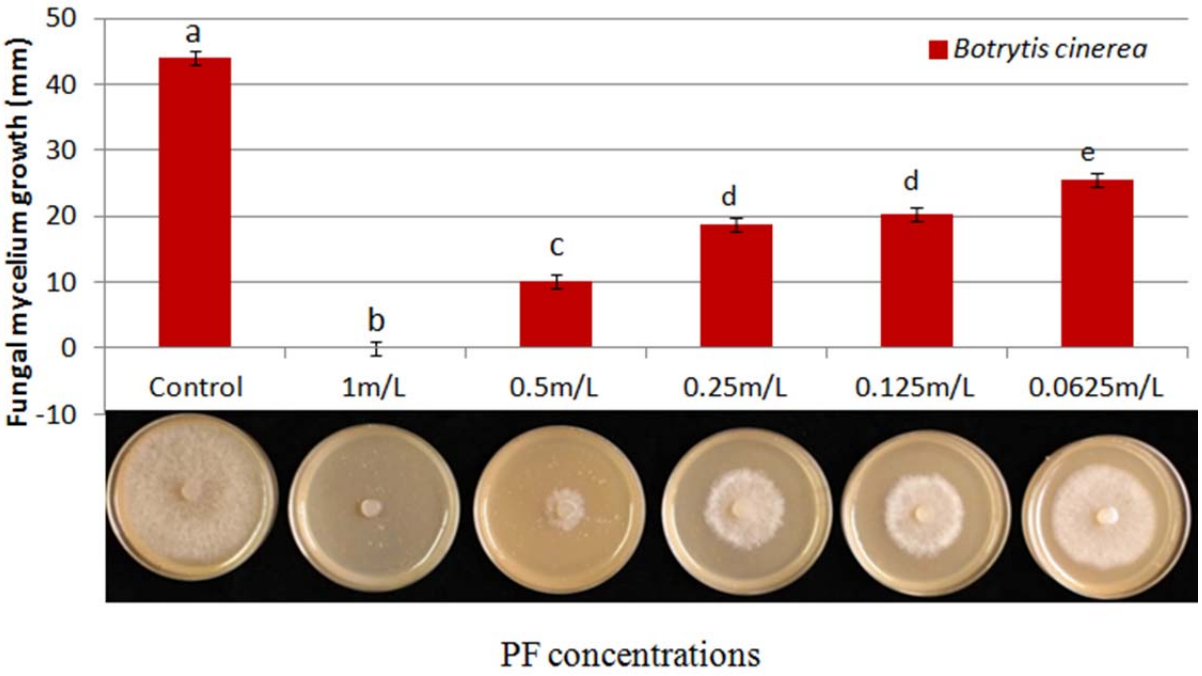

Figure 3. Mycelial growth of *Botrytis cinerea* (B03) on agar containing PF at five different concentrations.

Table 6. Effect of PF (1 ml/L) on mycelial growth of 33 pathogenic fungi 72 h after incubation at 23°C using an inhibition assay.

Fungal isolates	Isolates no.	Colony diameter (mm)*
Control (V8 only)	---	44.00[a]
Alternaria sp.	A04	00.00[e]
Alternaria sp.	A20	00.00[e]
Alternaria sp.	A22	00.00[e]
Botrytis sp.	B01	03.00[c]
Botrytis sp.	B02	00.00[e]
Botrytis sp.	B03	00.00[e]
Botrytis sp.	B04	00.00[e]
Botrytis sp.	B05	04.00[b]
Botrytis sp.	B06	00.00[e]
Botrytis sp.	B07	04.00[b]
Botrytis sp.	B08	00.00[e]
Botrytis sp.	B09	00.00[e]
Botrytis sp.	B10	00.00[e]
Colletotrichum sp.	Col1	00.00[e]
Fusarium sp.	F03	00.00[e]
Fusarium sp.	F07	02.00[d]
Fusarium sp.	F08	00.00[e]
Geotrichum sp.	G01	00.00[e]
Geotrichum sp.	G02	00.00[e]
Geotrichum sp.	G03	00.00[e]
Geotrichum sp.	G05	00.00[e]
Geotrichum sp.	G06	00.00[e]
Geotrichum sp.	G07	00.00[e]
Geotrichum sp.	G08	00.00[e]

Table 6. Contd.

Geotrichum sp.	G09	00.00^{e}
Mucor sp.	M01	00.00^{e}
Mucor sp.	M03	00.00^{e}
Mucor sp.	M04	00.00^{e}
Penicillium sp.	P07	00.00^{e}
Phoma sp.	Ph01	00.00^{e}
Phoma sp.	Ph02	00.00^{e}
Rhizopus sp.	R01	03.00^{c}
Stemphyllium sp.	S01	00.00^{e}

*Mean values followed by different letters within a column are significantly different according to Duncan's multiple range tests ($p \leq 0.05$).

growth of all isolated fungi but had no effect on the bacteria. This natural product is a potential alternative to synthetic fungicides in reducing postharvest gray mold disease.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Analysis of the energetic dynamism between solar and wind energy available in the south of Brazil

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Based on the concept of sustainability, which covers the energy issue as well as its rational use from natural resources, conventional sources do not meet the real requirements of the current production methods. In that context the west region of Paraná state in Brazil presents a vocation for developing projects that involve energy production from alternate sources such as biomass, water, wind energy and solar. Thus, the aim of this research was to verify the behavior of solar and wind sources in the city of Cascavel, Paraná, under different time series of a weather station, subjecting them to quantitative and qualitative analyses of their available energy, by using a statistical approach with Spearman's correlation coefficients (CC). Solar radiation and wind speed were observed. The correlation coefficients used in the analyses of behavior among alternative sources showed weak correlation with independence between variables in all parameters. Solar energy availability was proportionally more representative than wind availability in all periods assessed, mainly between inter seasonal averages.

Key words: Energy availability, renewable sources, correlation coefficients, clean energy.

INTRODUCTION

The region within 30° North and 30° South from the Equator (Asia, Africa and Latin America) has plenty of one or more renewable and clean energy sources, as solar, wind, water, biomass, geothermal and tidal. The issue is how to proceed in combining these resources with human needs (Ramakumar and Bigger, 1993).

Brazil has a significant share of renewable sources in its energy matrix, reaching 74.60% in 2014, which is above the global average of 19.70% in 2010, according to the International Energy Agency (IEA). The Brazilian economy is two times less polluting than the American, 1.3 times less than the European and 4 times less than the Chinese (MME-Ministry of Mines and Energy (Brazil) and EPE- Empresa de Pesquisa Energética, 2015). Hydropower is the main production source in the Brazilian energy matrix with 76.90%. Hydroelectric power plants are great energy providers, but in general terms they are limiting for a number of conditions concerning

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environmental, logistical and financial issues, having to depend on other forms of renewable energy to meet the world's needs with the same efficiency and sustainability (MME-Ministry of Mines and Energy (Brazil) and EPE-Empresa de Pesquisa Energética, 2015). The Brazilian aptitude for renewable energy options is potentially favorable, mainly with water, solar, wind and biomass.

Studies by (Tiba et al., 2000) in a time span of 40 years showed that the average daily global solar radiation in Brazil ranges from 8 to 22 MJ/m².day^{-1}. In 10 years of studies (Pereira et al., 2006), found that the southern region of Brazil has an inter annual average between 17 and 22 MJ/m².day^{-1}. In Paraná the annual average of daily global solar radiation is 16 MJ/m².day^{-1} (Tiba et al., 2000). In 2014 the installed capacity for wind generation in the country increased by 122.0%. The northeast region presents the country's largest wind energy production with installed capacity of electrical generation of 3.9 MW. The advancement of Brazilian wind generation is noticeable; the difference between 2013 and 2014 represented an increase of 85.60% (MME-Ministry of Mines and Energy (Brazil) and EPE-Empresa de Pesquisa Energética, 2015).

Alternative sources of energy can be supplied together. The solar and wind energy sources can be regarded as complementary in time, space or both (Beluco et al., 2008). Li et al. (2009) examined the correlation of wind and solar power in Australia for a whole year. Studies of (De Jong et al., 2013) were based on statistical data variables that defined the solar and wind resource and its correlation with the load curve between levels of hydroelectric reservoirs. According to (Notton et al., 2011), it is possible to analyze weather data in order to check the available energy from wind and solar sources using a correlation coefficient (CC) for a given period. Correlation analysis is the definition of a numerical direct relationship between two variables and Spearman's correlation coefficient can be used for data that do not have a standard, as well as for nonparametric data using only ranks, also called Spearman's rank coefficients; this method makes no assumption about the frequency distribution of variables (Shimakura, 2005).

In this context, the aim of this study was to analyze the behavior of alternative sources from solar irradiance and wind speed and direction, turning the hourly data inherent to these variables into different time series in order to describe in more detail the energy availability provided by solar and wind sources in Cascavel, PR.

MATERIALS AND METHODS

Availability of solar and wind energies

Solar energy

Several devices are used for measuring solar radiation and its components; the Campbell-Stokes heliograph measures the number of hours of sunshine or heat; the actinographer measures the total or diffuse solar radiation; the pyrheliometer measures the flow of direct solar radiation, and the pyranometer measures global radiation (Tiba et al., 2000). These measuring devices, according to (Martinazzo, 2004), meet the technical recommendations of the World Meteorological Organization (WMO) in what concerns to their installation and calibration, what makes them reliable and accurate, since each device requires specific care according to their purpose and limitations. The Angstrom relationship is the one existent between the daily global insolation and radiation. This method was established in 1924 and was modified by Prescott in 1940, and is currently known as the Angström-Prescott equation, defined by Equation 1, in accordance with (Suehrcke et al., 2013), in which the rate of daily global solar radiation H $[MJ.m^{-2}]$ by the daily global radiation at the top of the atmosphere $_{H0}$ $[MJ.m^{-2}]$ is equivalent to the sum of the linear regression coefficients $a + b$ $[dimensionless]$, being multiplied by the ratio between daily insolation n $[hours]$ and daytime duration n $[hours]$. The pyranometer was used to measure global solar radiation in a horizontal surface.

$$\frac{\mathrm{H}}{\mathrm{H_0}} = \mathrm{a} + \mathrm{b}\left(\frac{\mathrm{n}}{\mathrm{N}}\right) \quad (1)$$

Wind energy

According to (Masseran, 2015; Pinto, 2013), when the flow of air in motion is variable through time, it turns into potential wind energy P $[W.m^{-2}]$ (Equation 2). It is used in order to better describe the flow of wind power available, which in this situation is directly proportional to wind speed cubed v $[m.s^{-1}]$, the area of the airstream that has been measured at a perpendicular plane to wind speed direction A $[m^2]$ and air density $[kg.m^{-2}]$.

$$\mathrm{P} = \left(\frac{1}{2}\right).\rho.\mathrm{A}.\mathrm{v}^3 \quad (2)$$

Air density ρ is established according to local altitude z $[m]$, atmospheric pressure in relation to sea level P_0 $[kg.m^{-3}]$ and temperature T $[K]$ with the air specific constant R $[\frac{J}{K}mol]$ and gravity acceleration g $[m.s^{-2}]$, as shown in Equation 3 (Pinto, 2013).

$$\rho\,(\mathrm{z}) = \frac{\mathrm{P_o}}{\mathrm{RT}}\mathrm{e}^{-\frac{\mathrm{gz}}{\mathrm{RT}}} \quad (3)$$

Characterization of the area and obtainment of meteorological data

The obtainment of solar and wind energy data was provided by a meteorological station model Hobo U30, located at 18 meters high, at coordinates - 24° 59' 19" (S) and - 53° 26' 52" (W), 763 m above sea level, as shown in Figure 1. Data acquisition was performed automatically for a period of two years by means of a Datalogger.

Time series

The several types of weather variations are highly relevant for the use of solar and wind energy, considering daily, annual, seasonal and short-term variations, the latter in the case of wind speed (Macêdo and Pinho, 2002). After automatic data collection by the

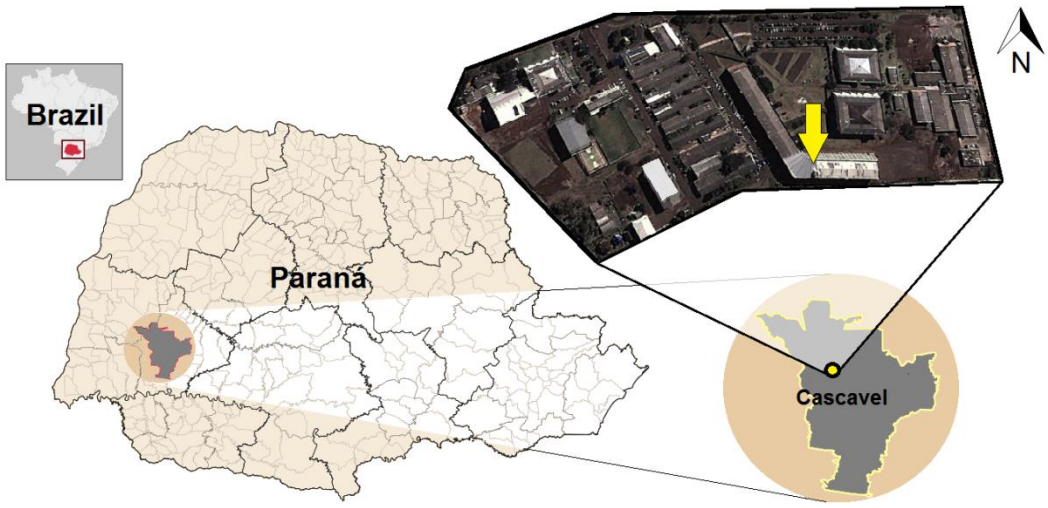

Figure 1. Map with the location of the meteorological station with automatic acquisition of solar radiation and wind speed data in UNIOESTE. Source: author.

Table 1. Spearman's rank coefficients.

Negative	Level	Positive
[-1]	Perfect	[1]
[-0.9 to -1]	Very Strong	[0.9 to 1]
[-0.6 to -0.9]	Strong	[0.6 to 0.9]
[-0.3 to -0.6]	Regular	[0.3 a 0.6]
[0 to -0.3]	Weak	[0 to 0,3]
[0]	Absent	[0]

Source: adapted from (Masseran, 2015).

weather station, the five-minute intervals were changed to hourly intervals. The variables of barometric pressure, ambient temperature, solar irradiance, wind speed and direction were processed into a spreadsheet. Mean and/or cumulative values were discussed in order to analyze the solar and wind energy availabilities occurring in these different variations of time, for a daily, monthly, yearly and hourly stationary discussion. Discussions related to the behavior of the time series, wind and solar availability and analysis of correlation coefficients are put separately to assess the variations observed in each series.

Energy availability data analysis

Posterior to obtaining the solar irradiation meteorological data, the available solar energy (ASE) [Wh] was calculated with the nth average of the data group n [$unit$] by multiplying the described average global radiation $l_h[Wh]$ described by Equation 4. The 5 min intervals were changed to hourly intervals by multiplying them by the coefficient demonstrated in Equation 5.

$$\mathrm{ASE} = \sum_{i=1}^{n} l_h \ \mathrm{x\,t} \quad (4)$$

$$\mathrm{t} = \frac{1}{12}\ \mathrm{hours} \quad (5)$$

For the available wind energy (AWE) [Wh], the nth average of the data group n [$unit$] was calculated by multiplying the average wind power described in Equation 6. The 5 min intervals were changed to hourly intervals by means of multiplying them by the coefficient demonstrated in Equation 7. The same was done to solar energy.

$$\mathrm{AWE} = \sum_{i=1}^{n} \mathrm{P\ x\,t} \quad (6)$$

$$\mathrm{t} = \frac{1}{12}\ \mathrm{hours} \quad (7)$$

Analysis of available energy correlation

Spearman's rank correlation coefficient ρ [$dimensionless$], expressed in Equation 8 (Andriotti, 2010) was used for verifying the correlation rank among variables, in which the differences among ranks with correspondent x and y [$numeral$] values and pair numbers of the n [$unit$] values are inserted. The correlation ranks of this coefficient's classification range from - 1 to 1, as shown in Table 1.

$$\rho = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{n\,(n^d - 1)} \quad (08)$$

RESULTS AND DISCUSSION

Interannual energy availabilities

The peak hours for solar and wind energy availability were concentrated at 10 and 12 o'clock, as shown in

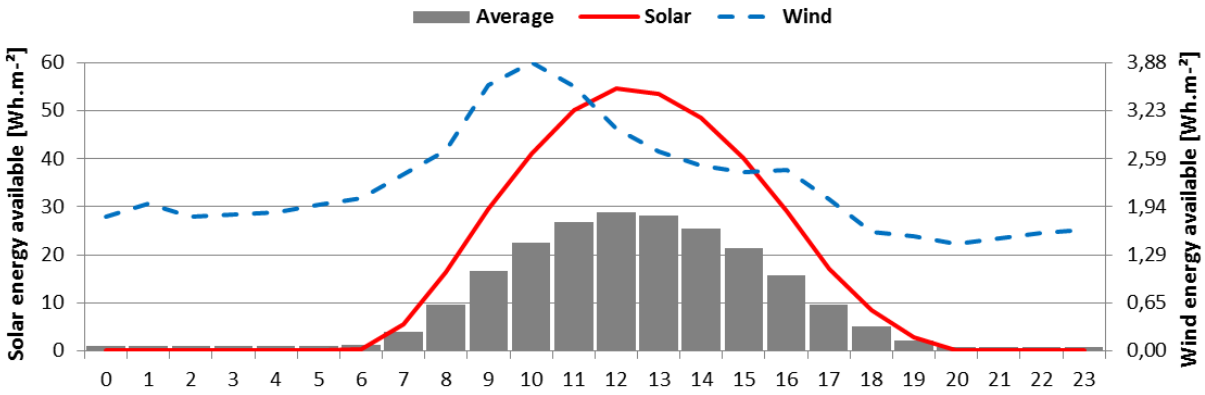

Figure 2. Availabilities energetic (solar and wind) hourly during a typical year.

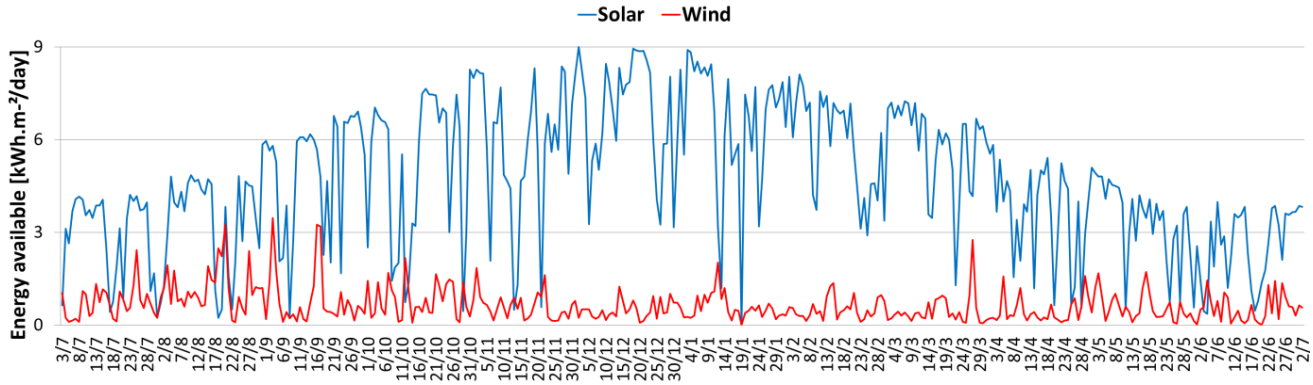

Figure 3. Availabilities energetic (solar and wind) during a typical year.

Figure 2. The daily temperature cycle, according to (Tubelis and Nascimento, 1986), reflects the solar irradiation variation throughout the day. The daily variations of radiation balance occur due to the sun daily path above the horizon, and during this moment of the day, the uneven warming of soil surface induces the ascending hot air to replace colder air masses, occasioning a thermal and gradient difference of pressure and generating a bigger flow of air masses. The hourly averages of energy availabilities always had solar energy with the highest potential during the year typical. The solar energy was 7.39 times superior to wind energy. The highest hourly averages of solar energy availabilities obtained were 54.67 $Wh.m^{-2}$ (Solar) and 3.88 $Wh.m^{-2}$ (wind).

The lowest daily average of solar energy availabilities during a typical year was 0.24 $Wh.m^{-2}$, and the highest 9.00 $Wh.m^{-2}$. The lowest daily average of wind energy availabilities was 0.00 $Wh.m^{-2}$and the highest 3.46 $Wh.m^{-2}$. The solar energy availability presented a typical behavior of the Sun's seasonal trajectory (Figure 3).

Interseasonal energy availabilities

The main characteristic related to the natural resources observed concern their diluted energetic nature, verified on seasonal periods (Nogueira, 2004). This peculiarity was noticed on the solar and wind energy availability verified between the seasons of the period observed, showing a higher solar potential during summer. A higher wind potential was verified during fall, showing a typically stochastic wind peculiarity, as seen in Figure 4. During winter the solar energy was 10.38 times superior to wind energy; in spring, 23.60 times superior; in summer, 11.92 times superior and in fall 5.53 times superior.

Minimum and maximum values were assessed for daily average availabilities among seasons. The lowest solar energy availabilities were 0.74, 1.81, 2.89 and 0.42 $kWh.m^{-2}$ and the highest were 6.43, 8.87, 8.60 and 6.43 0.42 $kWh.m^{-2}$ for winter, spring, summer and fall, respectively. The lowest wind energy availabilities were 0.00, 0.04, 0.03 and 0.03 $kWh.m^{-2}$ and the highest were 2.10, 1.08, 6.51 and 7.87 $kWh.m^{-2}$ for winter, spring,

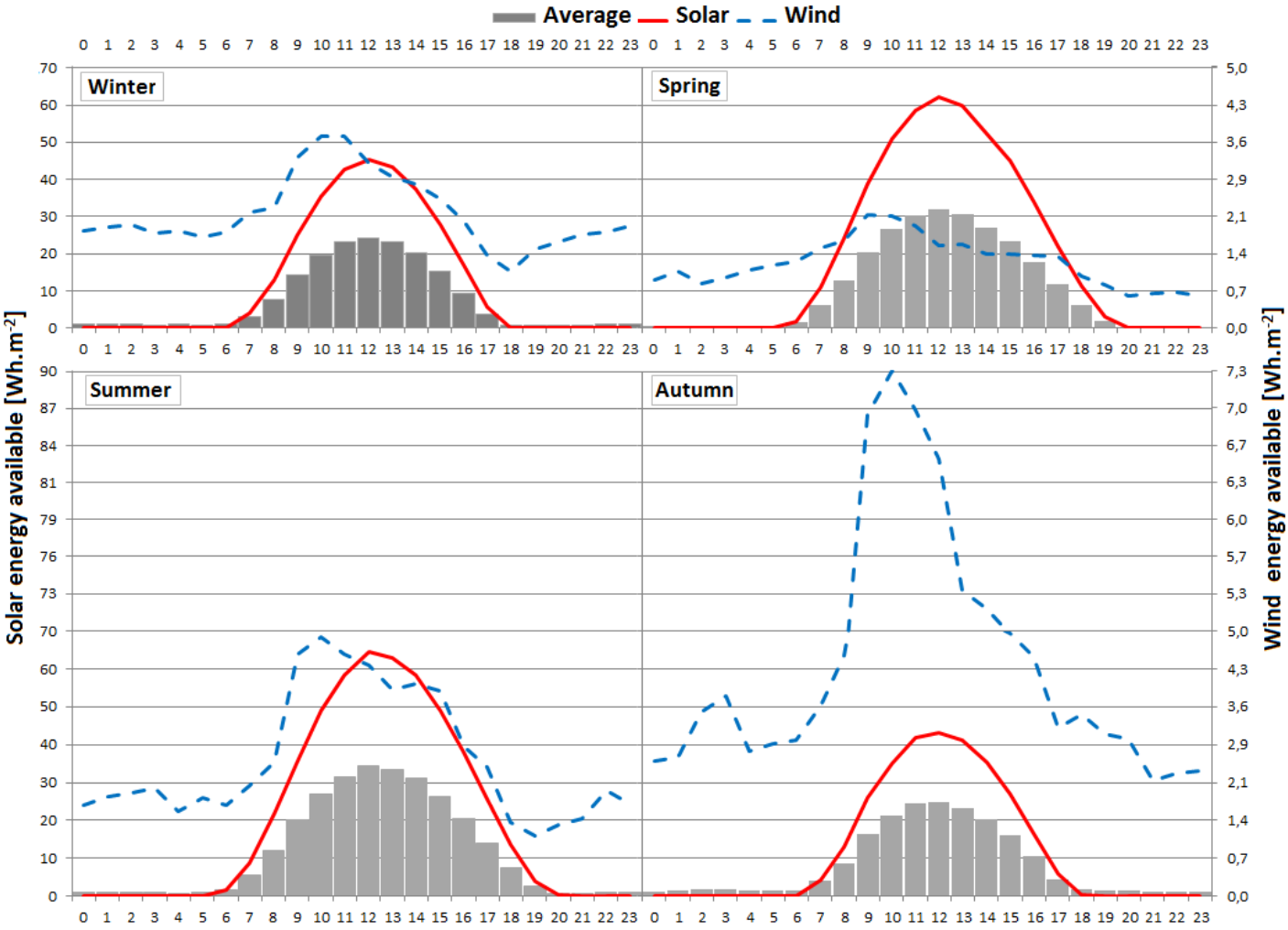

Figure 4. Daily averages of solar and wind energy availabilities between seasons.

summer and fall, respectively. The solar availability presented its highest values in the transition between spring and summer. According to Nobel (1983), wind regimes are seasonal, once the direction and speed are set accordingly to the intensity of radiation changes during seasons. However, the wind energy presented higher availability values in the end of summer and beginning of fall with the decrease of stationary radiation. The average availabilities among alternative sources were 0.58, 4.50, 5.57 and 0.49 kWh.m^{-2} in winter, spring, summer and fall, respectively, as seen in Figure 5.

Correlation coefficient (CC) analysis

The CC among energy availabilities for year 1 was negative and weak (- 0.07), in which independence among variables prevailed due to the existing variability between solar and wind amplitudes. In year 2, the correlation coefficient was negative and regular (- 0.32), mainly because of the sharp increase of wind variables in relation to solar variables, as shown in Figure 6, on the second part of this interval. The highest positive (0.32) correlation coefficient for the solar variable was found in year 1. In year 2 the lowest negative correlation coefficient found between wind and solar variables was (- 0.32). 40.00% of the coefficients on the matrix presented weak correlation levels and 20% presented regular levels (Table 2). The correlations between wind and solar variables among the seasons were positive and weak (0.17) in spring and (0.08) fall, and negative and weak (- 0.07) in winter and (- 0.05) summer. These coefficients show that energy availabilities have correlation independency between solar and wind variables during the seasons, as shown in Figure 7.

The highest correlation coefficient between wind and solar variables for stationary periods observed between

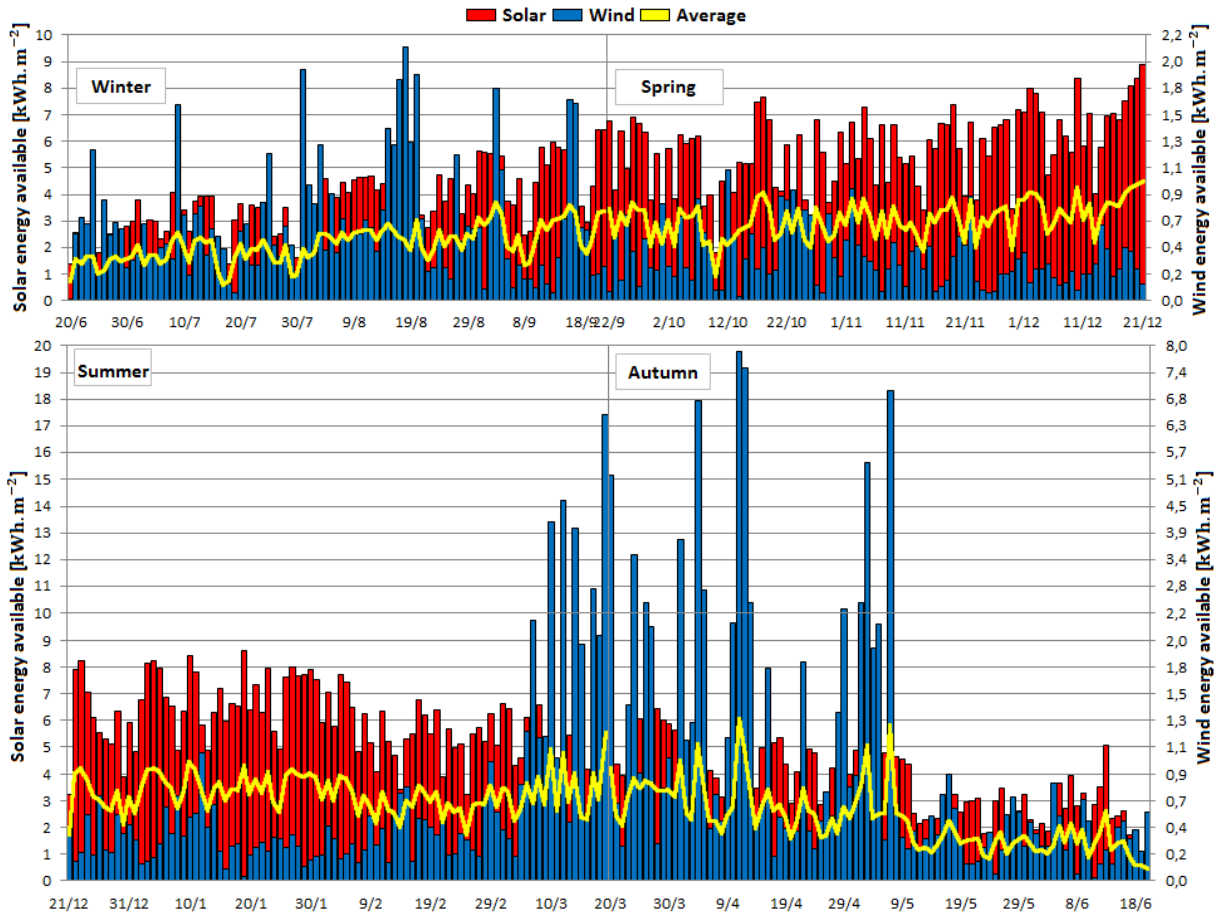

Figure 5. Daily averages of solar and wind energy availabilities among seasons.

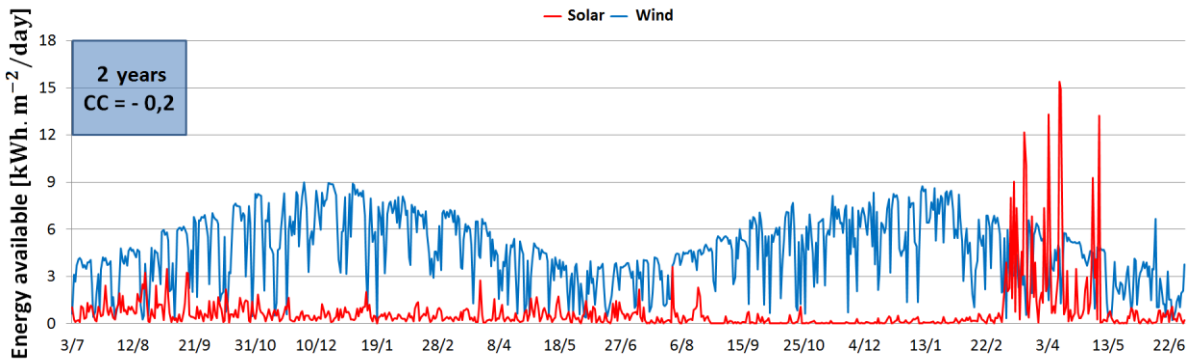

Figure 6. Solar and wind energy availability and CC for 2 year period.

winter and spring was regular (0.37) (Table 3), in which the value concerns to the most stable averages of wind availability. The lowest correlation coefficient considered weak (-0.24) was observed between summer and fall, in which the variability of the wind variable is inversely proportional. 69.44% of the coefficients on the matrix

Table 2. CC of the period averages 2 years of energy availability

Months		Jul		Aug		Sep		Oct		Nov		Dec		Jan		Feb		Mar		Apr		May		Jun	
	Variables	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W
Jul	*S	1																							
	**W	0,16	1																						
Aug	S	0,05	-0,21	1																					
	W	-0,02	0,02	-0,21	1																				
Sep	S	-0,10	0,14	0,15	0,00	1																			
	W	-0,03	0,17	-0,28	0,51	0,06	1																		
Oct	S	0,10	-0,04	-0,14	0,33	-0,29	0,25	1																	
	W	0,14	0,32	-0,24	0,45	0,17	0,39	-0,04	1																
Nov	S	-0,17	-0,06	-0,19	0,18	0,09	0,23	0,14	0,01	1															
	W	0,11	0,31	-0,32	0,54	0,10	0,50	0,10	0,57	0,15	1														
Dec	S	-0,18	0,23	-0,23	0,27	0,17	0,26	0,00	0,18	0,03	0,29	1													
	W	0,00	0,28	-0,14	0,37	0,26	0,50	0,03	0,52	0,09	0,57	0,17	1												
Jan	S	0,14	-0,23	0,17	-0,16	-0,11	-0,13	-0,02	-0,14	0,08	0,02	-0,17	0,01	1											
	W	0,18	0,46	-0,12	0,35	0,15	0,43	0,04	0,44	-0,06	0,61	0,16	0,52	-0,15	1										
Feb	S	-0,06	0,11	-0,13	0,48	0,02	0,39	-0,05	0,36	-0,04	0,33	0,30	0,32	-0,19	0,32	1									
	W	-0,18	0,14	-0,21	0,54	0,21	0,38	0,08	0,31	0,07	0,53	0,36	0,32	-0,21	0,39	0,37	1								
Mar	S	0,14	0,04	-0,09	0,29	-0,01	0,24	-0,04	0,46	-0,04	0,41	-0,07	0,24	0,06	0,31	0,27	0,08	1							
	W	-0,03	-0,25	0,28	-0,28	-0,03	-0,26	0,12	-0,56	0,12	-0,60	-0,01	-0,33	0,09	-0,43	-0,24	-0,33	-0,53	1						
Apr	S	-0,05	-0,21	-0,06	0,09	0,01	-0,02	0,24	-0,05	0,22	-0,07	0,12	-0,03	-0,04	-0,18	0,21	0,05	-0,29	0,19	1					
	W	-0,06	-0,10	0,11	-0,25	0,04	-0,36	-0,25	-0,46	0,02	-0,34	-0,28	-0,34	0,08	-0,41	-0,17	-0,13	-0,06	0,18	-0,12	1				
May	S	0,11	0,10	-0,17	0,10	-0,24	0,19	-0,17	0,20	-0,03	0,18	0,18	-0,01	0,06	0,12	0,33	0,12	0,33	-0,08	-0,17	-0,05	1			
	W	-0,23	0,30	-0,30	0,13	-0,23	0,20	0,16	0,04	0,08	0,15	0,10	0,06	0,03	0,16	0,12	-0,07	0,22	-0,28	-0,09	-0,17	0,15	1		
Jun	S	0,13	0,48	-0,12	0,03	0,18	0,06	-0,28	0,10	0,08	0,11	-0,11	0,06	-0,19	0,32	0,05	0,05	-0,04	-0,20	-0,25	0,21	0,04	0,08	1	
	W	0,08	-0,08	0,06	0,17	0,25	0,24	0,24	0,27	0,20	0,25	-0,03	0,25	-0,01	0,22	-0,04	0,11	0,25	-0,16	0,27	-0,02	-0,27	-0,11	-0,04	1

Level		%
Perfect		8,00
Very strong		0,00
Strong		0,33
Regular		18,00
Weak		73,00
Absent		0,66
Total		100,00

*Solar **Wind.

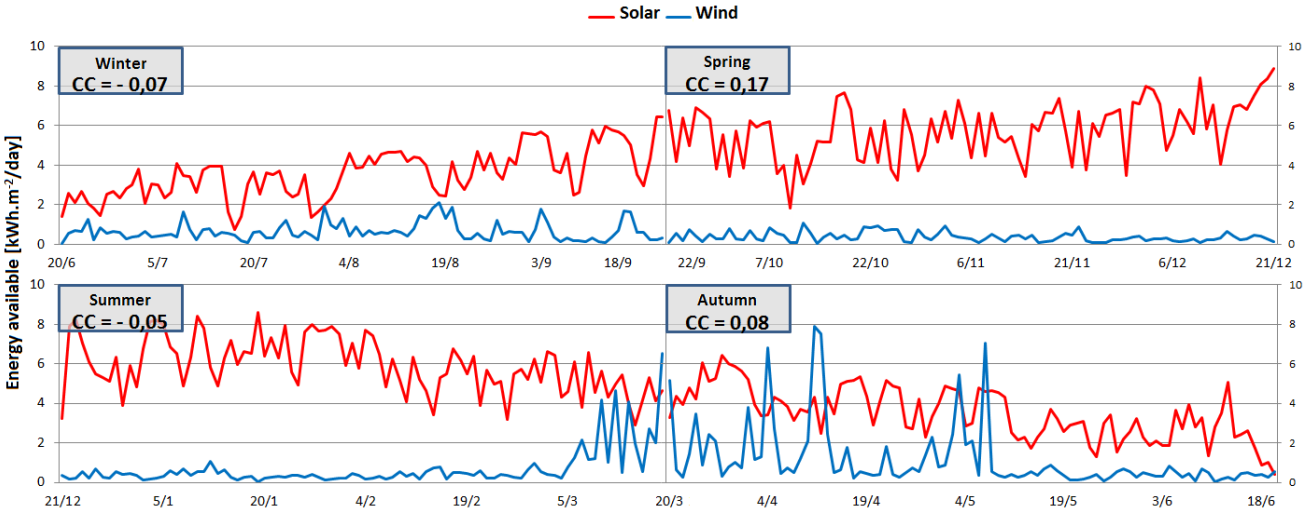

Figure 7. Solar and wind energy availabilities and CC during seasons.

Table 3. CC among stationary averages of energy availabilities.

Seasons		Winter		Spring		Summer		Autumn	
	Variables	Solar	Wind	Solar	Wind	Solar	Wind	Solar	Wind
Winter	Solar	1							
	Wind	-0,07	1						
Spring	Solar	0,13	-0,03	1					
	Wind	-0,10	0,37	0,17	1				
Summer	Solar	-0,03	0,14	-0,04	0,25	1			
	Wind	0,22	0,11	0,13	0,36	-0,05	1		
Autumn	Solar	-0,23	0,12	-0,05	0,13	0,28	0,00	1	
	Wind	-0,12	0,12	-0,14	-0,03	0,21	-0,24	0,08	1

Level		%
Perfect		22,22
Very Strong		0,00
Strong		0,00
Regular		5,55
Weak		69,44
Absent		2,77
Total		100,00

present weak correlation levels and 5.55% regular levels.

Conclusion

Wind availability is relatively low, does not present satisfactory energy complementarity, safeguarded the magnitudes among the alternative sources studied, such as the height observed of anemometer. Solar energy availability was proportionally more representative than wind availability in all periods assessed, mainly between interseasonal averages. The highest accumulated energy between seasons was observed in summer. The correlation coefficients used in the analyses of behavior among alternative sources showed weak correlation with independence between variables in all parameters.

Conflict of interests

The authors have not declared any conflict of interest

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Full Length Research Paper

Effect of combined biotic and abiotic stress on some physiological aspects and antioxidant enzymatic activity in mungbean (*Vigna radiate L.*)

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Stomata conductance (g_s), Shoot water content (SWC), chlorophyll pigments (chl a,b) and enzymes involved in anti-oxidant photo-protection were determined in two mungbean genotypes (Kawmay-1 and VC2010) under greenhouse conditions. The two genotypes were subjected to water deficit stress (20, 40 and 80% of field capacity) and two root-knot nematode (*Meloidogyne javanica*) infection levels (non-infected and infected at 15000 juveniles per pot). Both water deficit and nematode infection resulted to a fast decline in the chlorophyll pigments, g_s and SWC in both genotypes; however, VC2010 was recorded as being comparatively resistant. Increase in antioxidant enzymes activity was detected for superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and polyphenol oxidase (PPO) in both stresses, but this activity was more pronounced in water deficit stress than nematode infection, especially at 40% field capacity. APX and PPO production peaks recorded at 20% of irrigation in VC2010 were highest. This revealed that VC2010 genotype was tolerant to environmental stresses compared to Kawmay-1. It was conceived from the present study that water deficit stress significantly hampered the physiological representatives of plant health, while on the other hand enzymatic alterations to cope with the biotic and abiotic stresses in plants could be used for better tolerability and plant health. The results indicated that oxidative damage (ROS) produced under environmental stress can be minimized by increasing the antioxidant enzymatic activities in mungbean.

Key words: Enzymatic activities, Nematode infection, Mungbean, reactive oxygen species (ROS), stomata conductance, water deficit.

INTRODUCTION

Drought stress is characterized by reduction of water content, diminished leaf water potential and turgor loss, closure of stomata and decrease in cell enlargement and growth. Severe water stress may result in the arrest of photosynthesis, disturbance of metabolism and finally death of plant (Kaya et al., 2006; Jaleel et al., 2008). Water deficit is frequently the primary limiting factor for crop production. However, the response to stress depends on the intensity, rate and duration of exposure, as well as the plant growth stage (Hussain et al., 2004).

Drought problems for agricultural crops are on the increase with the rapid expansion of water stressed areas around the world. Limited water supplies and increasing demand sectors impose innovative and efficient water

employment in agriculture. Root-knot nematodes (*Meloidogyne* ssp.) are obligate endoparasites, which spend a greater part of their life cycle inside the host plant and parthenogenetically reproduce by mitotic divisions (Blaxter, 2003; Strajnar et al., 2009). More than 2,000 plants, comprising of major crops are attacked by *Meloidogyne* ssp., which is responsible for $157 billion (more than 50%) of overall damage caused by nematodes (that is, agricultural losses annually). Formation of root galls increases wilting reduces growth, nutrient and water uptake, which result to mineral deficiency and low yield. Among the various *Meloidogyne* ssp., *Meloidogyne javanica* is a potential threat to pulse crops. The biochemical and physiological activities of the host plants are hampered by *M. javanica* infection. In *Vigna radiata*, 23 to 49% yield losses has been reported to have resulted from *M. javanica* infection (Sharma et al., 2000).

Mungbean (*V. radiata* L.) originates from South Asia, and is being cultivated in the short rainy season in Southern Asia. Now, it has also been introduced in South East Asia, Austronesia, Africa, China, Egypt and South America (Lambridge and Godwin, 2006). Among the favourable characters of cultivating mungbean are: Short-term growth, nitrogen fixation capability, soil reinforcement and prevention of soil erosion (Hoorman et al., 2009). Mungbean seed is a rich source of protein (23.86%), carbohydrates (62.62%), minerals (K, P, Mg, and Ca), vitamins (C and A) and dietary fibre (Khattak et al., 2009). It enhances human body immunity, lowers the cholesterol level and protects against diabetes. Plant tolerability to various abiotic and biotic stresses at cellular, as well as at plant level is a complex issue. This complexity is due to interactions among various physiological, molecular, metabolic and morphological phenomena affecting growth and development under stress factors. Oxidative damage (ROS) produced under environmental stress) cause a serious problem in plants and can be minimized by increasing the antioxidant enzymatic activities (Hernandez et al., 2010). A systematic study is inevitable to understand the physiological response of mungbean under drought and nematode stresses. The present study was designed to investigate the physiological response of mungbean under drought-nematode coupled stress, under Saudi Arabian conditions.

MATERIALS AND METHODS

This greenhouse study was carried out in the Department of Botany and Microbiology, Faculty of Science, King Saud University, Riyadh, Saudi Arabia (24.710 N and 46.720 E) in 2012 to 2013.

Plant material

Seeds of two mungbean genotypes; Kawmay-1 and VC-2010, imported from Egypt and Thailand, respectively were surface sterilized with 0.1% sodium hypochlorite (NaOCl) for 5 min, washed with 0.1 M MgSO4 solution thrice and dried in open air. These seeds were sown in 30 cm plastic pots filled by soil-peat moss, with a 2:1 mixture; sterilized by steam under pressure at 126°C for 30 s. The experiment consisted of three replications and 36 pots arranged in a Randomized Complete Block Design (RCBD) under factorial arrangement with three factors: genotype (A), irrigation (B) and nematode infestation (C) (Gomez and Gomez., 1984). Pots were filled with recommended fertilizers and maintained to 6 seedlings per pot, two weeks after sowing.

Drought stress induction

Three weeks after sowing, the plants were arranged for imposing drought stress. Three irrigation levels (80, 40 and 20% of field capacity) were used as imposed drought stress in mungbean based on pretested field capacity of soil-peat moss mixture. All pots were irrigated weekly according to the treatments earlier given. After 45 days of nematode infection, data were recorded for the aforementioned parameters.

Stomata conductance (mmole/m^2/s)

Stomata conductance was measured using Leaf Porometer, Model: SC-1 (Decagon Devices USA). The measurements were taken between 10:00 to 11:30 a.m. using three leaves for each treatment.

Shoot water content measurement, SWC (%)

Shoot water contents (SWC) was measured by sampling three similar fully expended leaves per plot. Leaf samples were sealed in plastic bags, placed above ice in a cooler and transported to the lab for the determination of fresh weight. Leaf samples were dried at 70 ±5°C until constant weight was reached. SWC were calculated according to the following formula: SWC=[(FW-DW)/(FW)] × 100.

Chlorophyll contents (a and b)

Chlorophyll a and b contents were determined according to the technique described by Metzner et al. (1965). UV/Visible Spectrophotometer - LKB (Biochrom 4050) was used at wavelengths of 663 and 644 nm, chlorophyll contents were computed using the following equations:

Chlorophyll (a) = 10.3×OD663 - 0.918×O.D644 = ug/g
Chlorophyll (b) = 19.7×OD644 - 3.87×O.D663 = ug/g

Root-knot nematode inoculum

The *M. javanica* population was derived from single egg mass and cultured on tomato (*Solanum lycopersicum*) seedlings in a greenhouse. After 60 days of culturing, eggs were extracted from

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tomato by shaking infected roots for two minutes in 0.5% sodium hypochlorite (NaOCl) solution using an electric shaker (Hussey and Barker, 1973) followed by thorough washing under distilled water; a modified technique described by Mcclure et al. (1973). The eggs suspension was then incubated at 28 ± 2°C on cotton-wool filter to obtain freshly emerged juveniles. To avoid contamination and water evaporation, petri dishes were covered with glass lids. After 72 h of incubation, second stage juveniles were collected from suspension and stored under recommended conditions for further use (Walters and Barker, 1993). Number of juveniles was counted using counting dish, and 20 ml water suspension was prepared for 15,000 juveniles per pot. Suspension was poured uniformly in root zone 5 cm below the soil surface. Half of the mungbean pots were inoculated with root-knot nematodes four weeks after sowing, while the other half were kept non-inoculated as control.

Enzyme extraction and estimation

According to McCord and Fridovich (1969), 0.50 g of fresh leaves were collected and frozen in nitrogen liquid; then the sample was ground to make it soft. The sample was extracted by 6 ml of liquid composite of 50 mmol of potassium phosphate pH 7 + 0.1 mmol of EDTA + 4% of PVP + 0.2 mmol of ascorbic acid. Then, all were extracted in centrifuge at 1200 for 20 min. The pure liquid was kept in a refrigerator at 4°C till estimation.

Catalase (CAT)

CAT was determined by spectrophotometer according to Aebi (1984), where a mixture of 2 ml of enzyme extraction and 3 ml of interaction mixture (1.0 mmol Potassium phosphate pH 7 + 15 mmol of H_2O_2) were used.

Superoxide dismutase (SOD)

Superoxide dismutase was analyzed according to methodology described by Weisiger and Fridovich (1973), using viresitokrom and xanthine oxidase as a source of activation of SOD, followed by potassium cyanide for estimation of enzyme activity in hydrogen peroxide.

Ascorbate oxidase (ASO)

According to Saher et al. (2004), ASO enzyme activity was measured in a mixture of 50 mmol of potassium phosphate pH 7 + 1.5 mmol of ascorbic acid and 1.0 mmol of nitrogen peroxide. Then, the reading was done at 265 nm using a spectrophotometer.

Polyphenol oxidase (PPO)

The activity of enzyme was measured according to the method of Hernandez (2010), comprising 5 ml of catechol liquid with concentration 0.01 M + 2 ml of regulator liquid of phosphate and 0.50 ml of enzyme extraction, all were put in a test tube and measurements were taken after 3 min at 470 nm, using a spectrophotometer.

Statistical analysis

Analysis of variance (ANOVA) was performed by Statistical Analysis System (SAS, 2013) software. Means were compared using Fishers LSD analysis at 5% probability.

RESULTS AND DISCUSSION

According to Table 1 and Figure 1, the genotypic differences are significant for stomatal conductance (g_s) and SWC at 5% LSD while water deficit stress and nematode infection have been recorded as being highly significant (1%) for all mentioned parameters. Interaction between G and I stood significant for g_s, SWC, CAT and SOD, however, G*T recorded significant for stomatal conductance and SOD only, while all other parameters were non-significant. Irrigation by nematode interaction were computed as highly significant except for g_s and chlorophyll b. Stomatal conductance and shoot water contents exhibited almost similar trends, water deficit stress significantly hampered both parameters in descending order, from 80% < 40% < 20%. Infection by nematode also significantly reduced both parameters. VC2010 stood prominently resistant against water and nematode stress. G*I was recorded as significant for both; however, G*T and I*T were recorded as non-significant for stomatal conductance and SWC, respectively; whereas three way interaction was found as totally non-significant. Stomatal conductance (g_s) is an important and frequently varying plant parameter under both biotic and abiotic stresses and responds immediately. It regulates a number of physiological as well as biochemical process simultaneously e.g., rate of carbon assimilation, radiation absorption, transpiration. These findings were supported by the reports of Kaya (2006) and Jaleel (2008). photosynthetic pigments were restricted by water deficit stress, 20% irrigation reduced both chlorophyll a and b by more than 50% in comparison to control irrigation.

A similar trend was seen for nematode infection. However, no significant genetic differences were recorded. Genotype by Irrigation and Genotype by Nematode infection interactions were completely non-significant for both pigments; however, I*T was computed as highly significant for chlorophyll a but non-significant for chlorophyll b. Three factorial combined interactions were non-significant. Thalooth et al. (2006), Naresh et al. (2013) and Ahmed et al. (2009) reported almost similar results. These results indicated that stresses, drought and nematode infection have much effect on both genotypes. Water relations and chlorophyll content are very important in plant photosynthesis and hence crop productivity, so all physiological traits were affected by both stresses. These finding agree with many other authors (Sharma et al., 2000, Kaya et al., 2006; Jaleel et al., 2008) who found that severe water stress may result in the arrest of photosynthesis, disturbance of metabolism and finally death of plant.

Reactive oxygen species (ROS) produced under environmental stresses can cause a serious effect to plant metabolism and hence higher reduction in crop productivity. It is known that oxidative stress results from the disruption of cellular homeostasis of reactive oxygen

Table 1. Analysis of variance summary for mungbean genotypes under water deficit and *Meloidogyne javanica* stresses.

Sources of Variation	df	Mean squares (Summary)							
		Stomatal conductance	Shoot water contents	Chl_a	Chl_b	Catalase	Superoxide dismutase	Ascorbate oxidase	Polyphenol oxidase
		µmol/m^2/s	%	µg/g	µg/g	µmol/mg/min	µmol/mg/min	µmol/mg/min)	µmol/mg/min
Genotypes (G)	1	971.4**	0.11**	32100^{NS}	1002.7^{NS}	1372.7*	1995.1^{NS}	0.174^{NS}	1.1736^{NS}
Irrigation (I)	2	1668.0**	0.38**	46135**	29226.4**	3041.4**	56761.3**	166.661**	39.5836**
G*I	2	80.8*	0.001**	150^{NS}	4386.11^{NS}	45.4**	128.1*	0.257^{NS}	0.4053^{NS}
Disease (T)	1	1013.4**	0.22**	34627**	95069.4**	974.5**	12026.8**	18.063**	6.3336**
G*T	1	1013.4**	0.001^{NS}	30334^{NS}	69.4^{NS}	0.7^{NS}	676.0**	0.003^{NS}	0.0136^{NS}
I*T	2	40.4^{NS}	0.001**	50199**	1886.1^{NS}	60.3**	1509.8**	2.216**	0.2003*
G*I*T	2	37.0^{NS}	0.0001^{NS}	12234^{NS}	536.1^{NS}	51.2*	30.3^{NS}	0.001^{NS}	0.0053^{NS}

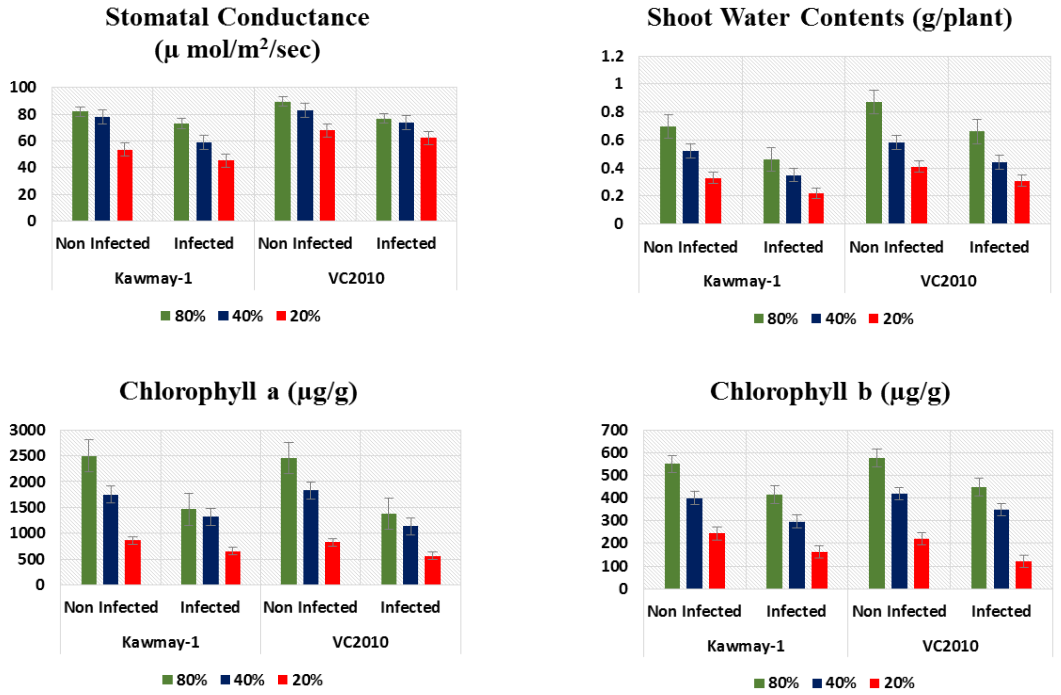

Figure 1. Physiological parameters of mungbean subjected to water deficit and *Meloidogyne javanica* infection.

Table 2. Antioxidant enzymatic activity of mungbean subjected to water deficit and *M. javanica* infection.

Genotype	Irrigation (%)	Nematode Infection	Catalase (CAT) (µ mol/mg/min)	Superoxide dismutase (SOD) (µ mol/mg/min)	Ascorbate Oxidase (APX) (µ mol/mg/min)	Polyphenol oxidase (PPO) (µ mol/mg/min)
Kawmay-1	80	Non-Infected	26.40±0.90	125.0±5.00	2.80±0.60	4.63±0.55
		Infected	18.40±2.00	119.3±1.52	2.23±0.15	3.90±0.30
	40	Non-Infected	58.60±1.80	270.0±5.00	6.83±0.35	7.33±0.45
		Infected	42.83±2.95	226.0±6.00	5.50±0.10	6.60±0.10
	20	Non-Infected	37.53±2.95	234.0±4.00	11.2±0.85	8.10±0.40
		Infected	29.23±0.95	200.0±12.0	8.93±0.35	6.93±0.15
VC2010	80	Non-Infected	31.50±0.80	138.3±6.50	3.23±0.55	4.90±0.30
		Infected	29.03±1.45	122.0±6.00	2.63±0.45	4.16±0.06
	40	Non-Infected	70.93±1.25	300.0±15.0	6.70±0.10	7.30±0.10
		Infected	60.20±5.90	238.0±2.00	5.30±0.40	6.73±0.35
	20	Non-Infected	56.40±4.20	261.3±5.50	11.4±0.10	8.83±0.06
		Infected	39.13±6.45	204.0±6.00	9.10±0.10	7.73±0.06

species (ROS) production. Reactive oxygen species accumulation induces oxidative damage of membrane lipids, nucleic acids, and proteins (Mitller, 2002).The response of antioxidant systems to drought stress has been widely studied in plants (Hernandez et al., 2001, 2010, Mittova et al., 2003, Gomez et al., 2004, BenAmor et al., 2006). In general, it is well accepted that plants with high levels of activity of the antioxidant systems, both constitutive and induced, have greater resistance to oxidative damage. Therefore, antioxidant enzymatic activity is known as a good mechanism can be used to protect plant from environmental stresses such as drought, heat, salinity and diseases infection (Sharma et al., 2000, Ahmed et al., 2002 and Hernandez et al., 2010). Our result indicated that CAT and SOD were recorded as being effected by nematode infection (Table 2). However, the enzymatic activity increased when irrigation level reduced from 80 to 40 but a reduction for 20% irrigation was noticed. APX and PPO were also found significantly reduced by nematode stress. Irrigation deficiency stress increased the APX and PPO activity as irrigation reduced from 80 to 20%, with the highest values recorded in 20%. This means that, under high stress, plants may increase the enzymatic activity to minimize the effect of oxidative damage by ROS produced under environmental stress. However, CV2010 mungbean genotype stood high for all four enzyme's activity recorded in this study, which indicated its tolerability to biotic and abiotic stresses. This finding may help for selection mungbean genotypes under arid climate of Saudi Arabia. Ahmed et al. (2002) reported similar results in a research conducted on mungbean plant.

Conclusions

The results illustrated that water deficit (Abiotic stress) may seriously damage the physiological activity and enzymatic performance in mungbean. Root-knot nematode (Biotic stress) may also significantly reduce the plant performance under particular conditions. However, it is considered that the present results confirm the relevance of induction of the antioxidant system to protect the plant against the oxidative damage under environmental stresses.

Also, genotypic differences for tolerance against biotic and abiotic stresses could be the potential inputs for variety development and agronomic practices. Further extensive research is necessary in order to understand and obtain deeper insights on the mechanism stress damage and biochemical changes behind physiological alterations.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effect of pesticides and micro-organisms on earthworm *Eisenia fetida* (Savigny, 1826)

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Earthworms might be limited in their activities on soil by pesticides used at important rates in agriculture and human pathogenic micro-organisms introduced in soil by excreta. This study using a modified toxicity filter paper contact test from Organisation for Economic Co-operation and Development (OECD), aimed at assessing the toxicity of six pesticides formulations and six micro-organisms on the earthworm *Eisenia fetida.* The study performed over 7 months at the laboratory QAIEA (University of Caen Normandie, France), analysed the mortality every 24 h for 96 h and at 96 h of *E. fetida* when exposing to pesticides and microbial suspensions. The statistical test used is the Student's t-test at the significant level of 0.05. No mortality of earthworms was observed when testing these pesticides at their recommended agricultural concentrations. Toxicity order from the highest to the lowest, based on LC_{50}, was Capiscol, Stratos Ultra Jardin, Polyvalent, Roundup GT Plus, Polyflor and KB Limace. Among tested micro-organisms, only *Enterobacter cloacae* (culture broth) and *Listeria monocytogenes* (culture broth and supernatant) generate mortalities of *E. fetida*. Finally, all these tested pesticides do not lead to *E. fetida* mortality if they are used at their recommended agricultural concentrations. Earthworms species *E. fetida* are also stressed by some micro-organisms. Furthermore, the filter paper contact test OECD might be used as a tool to evaluate the response of *E. fetida* to abiotic and biotic stresses.

Key words: Stress, toxicity, pesticides, micro-organisms.

INTRODUCTION

Among soil inhabiting organisms, earthworms are largely represented, counting more than 80% of soil

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invertebrate's biomass in tropical and temperate ecosystems. They can be divided into categories of epigeic, endogeic and anecic according to their burrowing abilities, feeding preferences and sizes (Mariana et al., 2001; Felten et al., 2009; Lalthanzara et al., 2011). Generally, they participate in soil aeration and water infiltration by creating burrows, increasing the nutrients content of the soil by incorporating litter into the soil, mixing soil minerals with organic material and producing on soil surface, their castings. These castings containing readily available and enriched form nutrients are from the large amount of the decomposed litter, manure and others organic matters they ingest. Regarding earthworms' capacity of affecting positively soil functioning through different mechanisms, they have been recognized as ecosystem engineers (Jones et al., 1994; Bartlett et al., 2010; Decëans et al., 2001). Thus, their abundance in soil is an indicator of the soil health and their activities are of great importance for agro-ecosystem sustainability.

However, these soil invertebrates of great interest are frequently facing different abiotic and biotic stresses in their living environment. They are threatened by biotic stresses (prey for platyhelminthes, amphibians, birds, mammals) and by abiotic stresses such as urbanism and intensive agriculture (Taboga, 1981; Carvalho, 2006; Fiore et al., 2004). Of all cases of threats to earthworms, intensive agriculture receives more attention because it requires important amounts of inputs including among others chemical pesticides.

Pesticides get to the soil either by direct application on the soil or runoff from foliar spraying, all in accordance with the manufacturer's instructions. Chemical stress caused by pesticides on earthworms are located at all biological levels such as physiology, biochemistry and genetic (Pelosi et al., 2014; Correia and Moreira, 2010). Beyond their impacts on earthworms, pesticides adversely affect humans, animals and soil organisms by contaminating environments such as air, soil and groundwater (Hussain et al., 2009; Bolognesi and Merlo, 2011; Barnhoorn et al., 2015). In a bid to guaranty food security for a growing earth's population estimated in 2050 to about 9 billion people (FAO, 2011), new formulations of pesticides and other chemical agricultural inputs are continuously manufactured for controlling diseases of comestible plants. Therefore, increasing information focusing on the effect of the pesticides formulations on earthworms is of great importance in the protection of soil biodiversity.

Besides the presence of pesticides in the soil, micro-organisms responsible for many severe diseases may also contaminate the soil through infected excreta of animals and humans and therefore, be in earthworm's surroundings. The biological interactions in soil between human pathogen micro-organisms and earthworms are indirectly elucidated by assessing the antibacterial properties of earthworms' coelomic fluid or its extract (Bhattacharjee and Ghosh, 2015; Li et al., 2011). On the other hand, earthworms usually take part in different interactions, soil living organisms have with one another (Pimm, 1982; Russell et al., 1985). Better knowledge of the earthworms/micro-organisms interactions is useful in environmental protection (e.g. use of earthworms in biological control of pests and in sewage treatment).

Eisenia fetida is a favorite worm species for composting and is frequently used as a biological monitor for testing the effects of contaminants on soil biota (Das Gupta et al., 2011; OECD, 1984; Garg et al., 2006). This study is cast within the framework of protecting earthworms that are important members of soil biodiversity and exploiting their ecosystem services for the environmental protection and repression of phytopathogenic micro-organisms. Its objective is to give an overview of stresses related to pesticide formulations and micro-organisms, that earthworms are facing in their living environment. Specifically, this study permitted assessment of the toxicity of six pesticides formulations (Round-up GT Plus, Stratos Ultra Jardin, Capiscol, Polyvalent, Polyflor and KB Limace) and six micro-organisms (*Geotrichum candidum* ATCC 203407, *Escherichia coli* UCMA 10579, *Enterobacter cloacae* UCMA 10580, *Listeria monocytogenes* UCMA 6115, *Salmonella typhi* UCMA 10598 and *Staphylococcus aureus* UCMA 6834) on *E. fetida*.

MATERIALS AND METHODS

Earthworms

Earthworms used in this study belong to the epigeic species *E. fetida*. They were purchased from Vers La Terre (Pezanas, France) and breeded at the analysis laboratory (University of Caen Normandie, France) with the help of a vermicomposting system (Worm Café). The vermicomposting was started with 1 kg of earthworms and carried out according to the manufacturer's instructions (VerlaTerre, 2016). At the composting set up, the vermicomposting system possessed two plates with one (the work plate) containing earthworms and coconut fibre. The coconut fibre was used as bedding for earthworms which was obtained by fully soaking a coconut fibre block (from Ceylon Garden Coir) in 6 to 7 L of tap water. Two handfuls of kitchen wastes (vegetable products, eggshells and coffee grounds) were placed three times every week on the coconut fibre. Moistened paper towel was put every week in the work plate to provide earthworms with fibres. The vermicomposting system was kept at room temperature (18 ± 2°C). From the third month of composting, adult earthworms (weighing between 300 and 600 mg) with well-developed clitella, were taken from the composting system and kept in fast for 3 h in the dark, at room temperature before use for toxicological assessment (OECD, 1984).

Pesticides

Six pesticides formulations Round-up GT Plus, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace from four different chemical classes were tested (Table 1). Their active substances are involved in widely used pesticides in agriculture field (Anonymous 1).

Table 1. Characteristics of used pesticides.

Pesticide	Nature	Active substance	Recommended agricultural dose	Corresponding area (m^2)	Manufacturing company
Round-up GT plus	Herbicide	Salt of glyphosate isopropylamin (607 g/L) equivalent to glyphosate acid (450 g/L)	20 to 40 mL/3L	80	Monsanto
Stratos Ultra Jardin	Herbicide	Cycloxydim (100 g/L)	2 to 4 mL/L	10	Fertiligène
Polyflor	Fungicide	Propiconazole (5 g/L)	10 mL/L	10	Syngenta Agro SAS
Capiscol	Fungicide	Azoxystrobin (250 g/L)	0.8 to 1 mL/L	10	Syngenta Agro SAS
Polyvalent	Insecticide	Deltamethrin (15 g/L)	2.5 to 4 mL/5 L	50	Bayer SAS
KB Limace Appat Granulé	Anti-slug	Metaldehyde (5%)	7 g	10	Fertiligène

Micro-organisms and their culture conditions

The fungus, *G. candidum* ATCC 203407 and bacteria *E. coli* UCMA 10579, *E. cloacae* UCMA 10580, *L. monocytogenes* UCMA 6115, *S. typhi* UCMA 10598 and *S. aureus* UCMA 6834 used in this study have been provided by the CONOBIAL (Conservatoire Normand de la microBiodiversité Alimentaire, Université de Caen Normandie, France). *G. candidum* ATCC 203407 was grown on Malt Extract Broth (MEB) at 25°C for 48 h (Naz et al., 2013) and bacterial strains were cultivated on liquid medium Luria-Bertani (LB) at 37°C for 48 h according to Rahman et al. (2012) with some modifications. These micro-organisms were incubated with shaking (120 rpm).

Acute toxicity test

The contact filter paper test of OECD (1984) was, with some modifications, used to assess the acute toxicity of pesticides, micro-organisms suspensions and their supernatants on *E. fetida*. Decimal dilutions were firstly performed for each type of product, in order to determine a range of concentrations in which a 0–100% mortality of the earthworms was obtained. The testing suspensions were also assessed at non-diluted concentrations. Concerning pesticides of which dilutions covered the Recommended Agricultural Dose (RAD), close dilutions (20, 40, 60 and 80% ratios of decimal dilutions) inside the 0–100% mortality interval were then carried out. The RAD of the used pesticides correspond to the dilutions and concentrations based on their active substances, respectively 6.6×10^{-3} to 1.3×10^{-2} and 2.97 to 5.85 g/L of glyphosate acid for Round-up GT Plus, (2 to 4) $\times 10^{-3}$ and 0.2 to 0.4 g/L of cycloxidim for Stratos Ultra Jardin, 10^{-2} and 0.05 g/L of propiconazole for Polyfor, 8×10^{-4} to 10^{-3} and 0.2 to 0.25 g/L of azoxystrobin for Capiscol, (5 to 8) $\times 10^{-4}$ and 0.0075 to 0.012 g/L of deltamethrin for Polyvalent and 10^{0} and 5% of metaldehyde for KB Limace. For micro-organisms suspensions and their supernatants, close dilutions were at 50% ratio of decimal dilutions. All dilutions were performed using peptone water (PW) composed of 1 g/L of peptone (polypeptone AES). PW and the growth medium LB were used as control. Micro-organisms suspensions included (i) stationary phase micro-organisms grown in liquid medium LB (culture broth) and, (ii) their washed cells. Washed cells were obtained by washing twice with peptone water. Firstly, culture of stationary phase micro-organisms was vortexed and centrifuged for 10 min at 7000 g. The supernatant was discarded and the pellet was retained for the washing steps. For the first washing, 10 mL of peptone water were added to the early stage pellet. The mix was vortexed and centrifuged for 10 min at 7000 g. The supernatant was discarded and the pellet was retained. Washing process was run again with the pellet from first washing. After this step (second washing), the pellet was suspended in 10 mL of peptone water by vortexing and this last mix was used as washed cells. Supernatants were obtained by centrifuging cultures of stationary phase micro-organisms at 7000 g for 10 min.

As a modified protocol of OECD (1984), a 10 cm diameter circular piece of filter paper (Fioroni, Paris, France) was placed in a 9-cm Petri dish and moistened either with 970 µL of each concentration of pesticides or 990 µL for micro-organisms suspensions and their supernatants. One earthworm was placed on this Petri dish in the contact with the moistened filter and a 10 µL quantity of testing suspension was spread inside the lid of dish. The dish was incubated in the dark at room temperature (18 ± 2°C) for 96 h and earthworm status (alive or dead) was recorded every 24 h. An earthworm was considered dead if it failed to respond to a gentle mechanical touch on the front end. The alive and dead statuses of earthworms are designated by the numbers 1 and 0, respectively. The toxicity test on *E. fetida* of each dilution of tested suspensions was carried out in triplicate (three living earthworms used) per experiment. At the beginning of the experiment (t = 0 h), the 3 living earthworms correspond to the number 3 and account for 100% of *E. fetida* survival. The percentage of *E. fetida* survival every 24 h is determined as follows: Percentage of *E. fetida* survival at time t = 100 x N at time t / N at time 0 h, with N representing the number of living *E. fetida*. Experiments were repeated at least 6 times for each dilution or concentration of tested suspensions and control suspensions LB and PW. Finally, 18 earthworms were used for assessing the toxicity of each concentration of tested suspensions (pesticides and micro-organisms) and control suspensions (LB and PW).

Statistical analyses

All statistical analyses were performed using Statisca 7 (StatSoft Inc, Tulsa, USA). The comparison between, (i) the effects average on *E. fetida* of each micro-organism suspension and its supernatant and, (ii) that of the control LB was carried out by a Student's t-test at the significant level of 0.05. On the basis of their lethal median concentration (LC_{50}) value, the toxicity levels of pesticides were compared by Anova ($p < 0.05$) and these pesticides were classified as being supertoxic (< 1 µg cm^{-2}), extremely toxic (1 - 10 µg cm^{-2}), very toxic (10 - 100 µg cm^{-2}), moderately toxic (100 - 1000 µg cm^{-2}) or relatively nontoxic (>1000 µg cm^{-2}) (Roberts and Dorough, 1984).

RESULTS AND DISCUSSION

Toxicity of pesticides

The toxicity to *E. fetida* of agricultural applications of pesticides Round-up GT plus, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace is

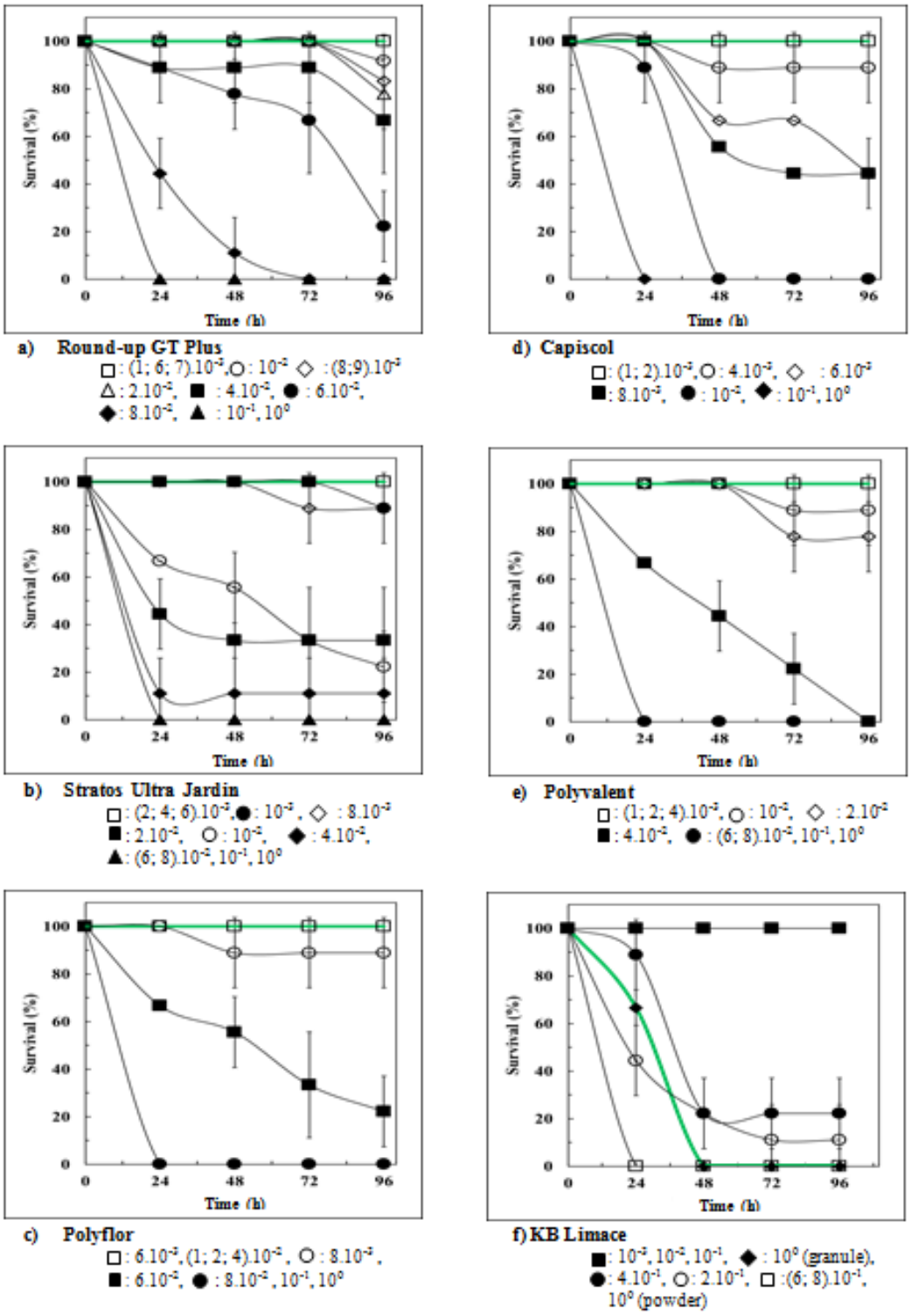

Figure 1. Survival of *Eisenia fetida* as function of time at different concentrations of pesticides (v/v: Round-up GT Plus, Stratos Ultra Jardin, Polyflor, Capiscol and Polyvalent; w/v: KB Limace; green curve: RAD).

presented in Figures 1, 2 and 3. The percentage of *E. fetida* survival decrease during time with increasing of overall pesticides concentrations (Figure 1). For KB Limace particularly, this proportionality principle is not

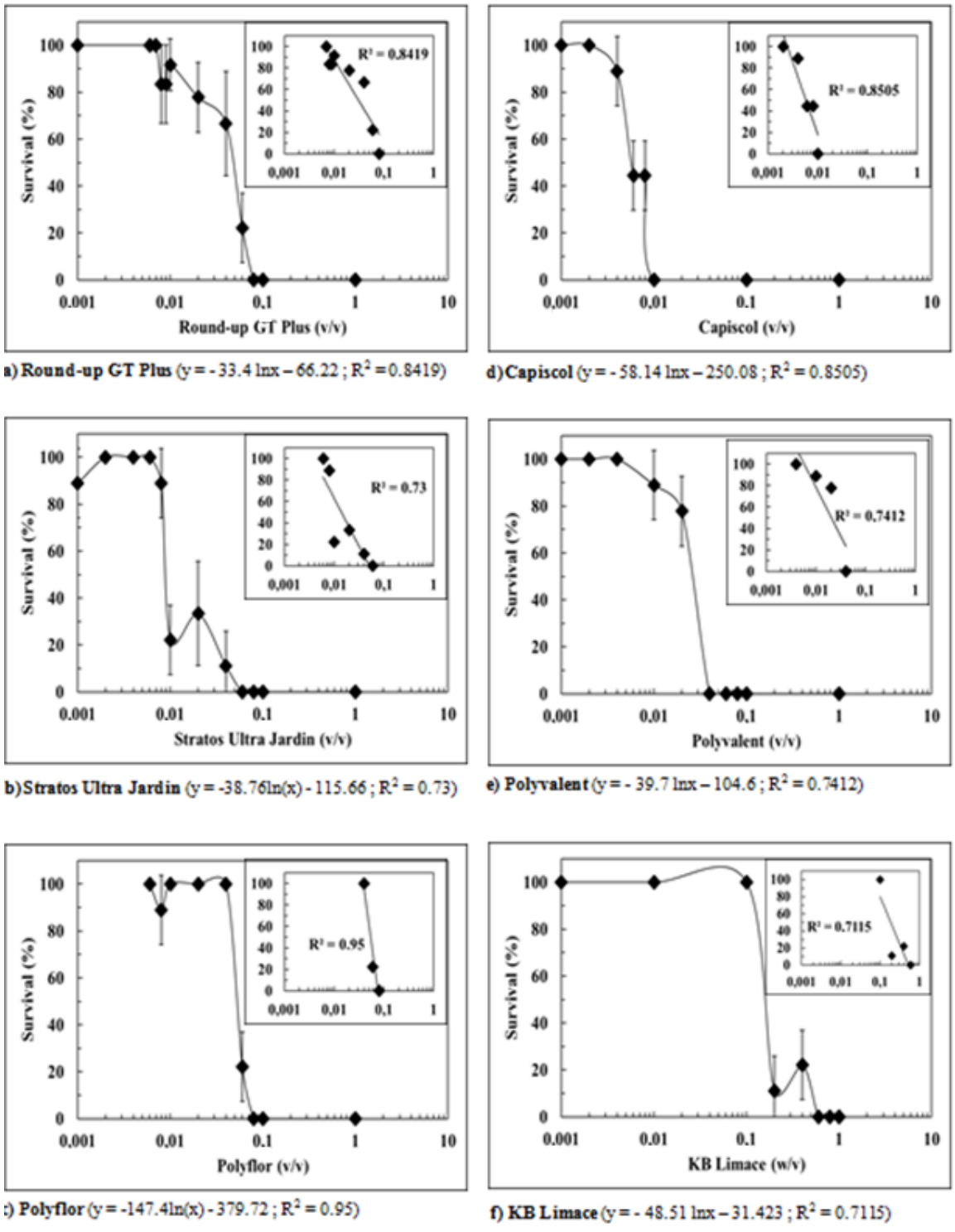

Figure 2. Survival of *Eisenia fetida* as a function of pesticides concentrations, over 96 h exposure.

always observed because of the granulated form of this pesticide formulation. In fact, this form does not enable a continuous contact between used earthworm and this pesticide, and consequently generate a biased estimation of its toxic effect. For all tested pesticides, no mortalities of earthworms were observed when exposed over 96 h at their Recommended Agricultural Dose (RAD) (Table 1, Figure 2). Concerning Round-up GT Plus, the toxicity result on *E. fetida* from its RAD value (2.97 to 5.85 g/L of glyphosate acid) to lower concentrations, is in accord with

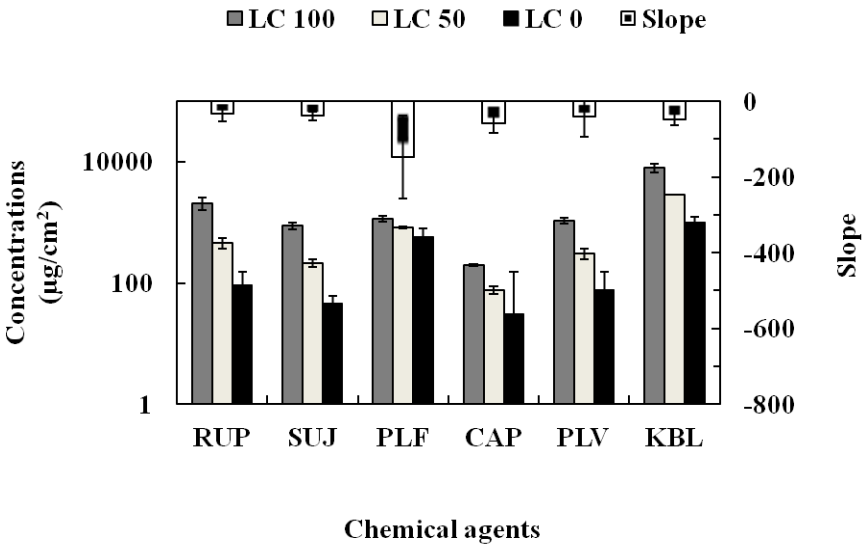

Figure 3. Pesticides toxicity on earthworm *Eisenia fetida* [RUP: Round-up GT Plus; SUJ: Stratos Ultra Jardin; PLF: Polyflor; CAP: Capiscol; PLV: Polyvalent; KBL: KB Limace; LC_{100}: Minimal concentration leading to 100% mortality; LC_{50}: Concentration leading to 50% mortality; LC_0: Maximal concentration leading to 0% mortality; Slope indicates the variation of earthworms survival (%) per dilution unit inside the [LC_0; LC_{100}] interval].

Correia and Moreira (2010) indicating no mortality for the same earthworm specie exposed to 1-1,000 mg/kg of glyphosate in soil. These data obtained after exposure to RAD concentrations from the filter paper contact toxicity test (described as an initial screen toxicity) showing no mortality of *E. fetida* after a 96 h exposure to pesticides, indicate that these pesticides generate mild or moderate stress on earthworms in soil (OECD, 1984). However, their use at important rates (e.g. in intensification of agriculture or for controlling pest in small scale or handicraft agriculture in undeveloped countries) could lead, through a runoff process from treated agricultural fields, to an acute and chronic poisoning in terrestrial and aquatic organisms. For instance, concerning Capiscol, the active substance azoxystrobin generates a risk for water quality and is recognized to be toxic for aquatic organisms (Rodrigues et al., 2013; Olsvik et al., 2010). Indeed, its RAD assessed in this study, equivalent to 0.2 to 0.25 g/L of azoxystrobin is several folds higher than 0.026 µg/L which is an environmental concentration value of azoxystrobin that induces a decrease of population in the cladoceran specie *Daphnia magna* after exposure (Warming et al., 2009). Other works showed that propiconazole (active substance of the formulation Polyflor) has low mobility and high adsorption in soil rich in organic matter so that it accumulates in top layers of soil (Thorstensen et al., 2001; Khairatul et al., 2013; Kim et al., 2002). Especially, some earthworms preferred as food by a wide range of animal, could be potential sources of pesticides intoxication by accumulating these chemical compounds (Hankard et al., 2004). This may result through food chain in pesticides bioaccumulation in terrestrial and aquatic organisms (Senthilkumar et al., 2001). On the other hand, the use at important rates of pesticides in agricultural fields could have long-term effects on non-target organisms (earthworms and others soil inhabiting organisms). These effects not only resulting in earthworms death, could disrupt or modify their activities and metabolisms (genes expression, physiology, behavior, population density) (Pelosi et al., 2011; Correia and Moreira, 2010; Contardo-Jara et al., 2009). A 96 h exposure of *E. fetida* to tested pesticides (Figure 2) showed that the range (LC_0 to LC_{100}) of highest and lowest pesticides concentrations causing respectively no mortality and 100% mortality of the studied earthworms population were 91.56 to 2090.62 µg/cm^2, 45.78 to 885.08 µg/cm^2, 579.88 to 1159.76 µg/cm^2, 30.52 to 198.38 µg/cm^2, 76.3 to 1083.46 µg/cm^2, 1007.16 to 7980.98 µg/cm^2 for Round-up, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace, respectively. The RAD values ranges of Round-up GT Plus (91.56 to 198.38 µg/cm^2) and Stratos Ultra Jardin (30.52 to 61.04 µg/cm^2) being close or including their respective LC_0 values, reinforce the thesis of non-use at important rates of these pesticides. These data constitute helpful information for database about chemical toxicity and agro-industry pesticides.

Toxicological profile of the pesticides

Unlike KB Limace, the LC_{50} after a 96 h exposure of the

Table 2. Survival percentage during time of *E. fetida* exposed to micro-organisms suspensions and their supernatant

Controls	Dilutions	Time (h)					
		0		24		48	
		CM	CM	CM	CM	CM	CM
PW		100	100	100	100	100	100
LB							
	10^0	100	100	77.77 ± 22.22 [a]	77.77 ± 22.22 [a]	0	0
	5.10^{-1}	100	100	100 [a]	100 [a]	33.33 ± 23.56 [a]	33.33 ± 23.56 [a]
Micro-organisms		**CB**	**S**	**CB**	**S**	**CB**	**S**
G. candidum	10^0	100	100	100	100	100	100
E. coli	10^0	100	100	44.44 ± 19.24 [a]	77.77 ± 19.24 [a]	0	0
	5.10^{-1}	100	100	88.88 ± 19.24 [a]	100 [a]	44.44 ± 19.24 [a]	0 [a]
E. cloacae	10^0	100	100	11.11 ± 19.24 [b]	11.11 ± 19.24 [b]	0	0
	5.10^{-1}	100	100	88.88 ± 19.24 [a]	77.77 ± 19.24 [b]	11.11 ± 19.24 [a]	0 [a]
L. monocytogenes	10^0	100	100	11.11 ± 19.24 [b]	100 [a]	0	0
	5.10^{-1}	100	100	77.77 ± 19.24 [b]	100 [a]	11.11 ± 19.24 [a]	0 [a]
S. typhi	10^0	100	100	66.66 ± 57.73 [a]	77.77 ± 38.49 [a]	0	0
	5.10^{-1}	100	100	100 [a]	100 [a]	11.11 ± 38.49 [a]	0 [a]
S. aureus	10^0	100	100	44.44 ± 19.24 [a]	33.33 ± 33.33 [a]	0	0
	5.10^{-1}	100	100	100 [a]	88.88 ± 19.24 [a]	22.22 ± 33.33 [a]	0 [a]

CB: Culture Broth; CM: Culture Medium; S: Supernatant; LB: Luria-Bertani; PW: Peptone Water. For same dilution between the control suspension and a given tested microbial suspension, values with the same letter in the same column are statistically ($p < 0.05$) equivalent.

tested pesticides are all higher than their RAD. According to the toxicological classification of chemicals using their LC_{50} values (Roberts and Dorough, 1984), the toxicological profile of these pesticides used on *E. fetida* are the following ones from the highest to the lowest. Capiscol (76.3 µg/cm^2) is classified as a very toxic chemical, Stratos Ultra Jardin (213.64 µg/cm^2), Polyvalent (305.2 µg/cm^2), Round-up GT Plus (457.8 µg/cm^2) and Polyflor (824.04 µg/cm^2) are considered as moderately toxic chemicals and KB Limace (2828.36 µg/cm^2) is in the range of relatively nontoxic chemicals (Figure 3). This comparative of chemical stress from a 96 h exposure to *E. fetida* shows that the toxicity of a given pesticide is not related to its nature (be insecticide, fungicide, herbicide or anti-slug). The 96 h LC_{50} value of Polyvalent (15 g/L or 1.5% of deltamethrin), is equal to the 48 h LC_{50} value of deltamethrin 98.0% purity (327.8 µg/cm^2) on the earthworm specie *E. fetida* (Kim et al., 2002). This indicates the existence in the mixture Polyvalent, of many stressors on earthworms. Also, Wang et al. (2012) found 566.1 µg/cm^2 as LC_{50} value when assessing the acute toxicity of glyphosate (85% purity) on *E. fetida.* This value being greater than the one of Round-up GT Plus, suggests that Round-up GT Plus is more toxic than the glyphosate (85%). Indeed, the Round-up GT Plus used in this study containing 45% of glyphosate acid, its toxicity on *E. fetida* may be greatly caused by the chemical additives or the synergetic effects of the chemical mixture in the formulation. Similar effects on reproduction in Zebrafish (*Danio rerio*) have been demonstrated with glyphosate (analytical grade) and its formulation Round-up GC liquid (120 g/L of glyphosate acid) (Webster et al., 2014). Works by Tsui and Chu on different organisms (bacteria, microalgae, protozoa and crustaceans) indicated higher toxicity for the polyoxyethylene amine (POEA: surfactant included in the Round-up formulation) than the Round-up (formulation) and followed by the glyphosate acid and glyphosate isopropylamine (active substances) (Tsui and Chu, 2003). It is observed that Polyvalent and Round-up GT Plus present more ecotoxicological relevances than their active substances (in comparison to others works) and regarding the increasing use of mixed pesticides in agriculture, due to their high efficiency, there is a need for a better ecotoxicological risk assessment of manufactured pesticides, to do not focus only on the toxicity of the active substance. Hence, earthworms having all their integrity could act properly for the well-being of environment.

Toxicity of micro-organisms

Data from the toxicity test on *E. fetida* with micro-organisms suspensions over 96 h are presented in the Table 2. These data indicated no effect of *G. candidum* culture broth, washed cells of all tested bacteria and peptone water (control) on *E. fetida* survival. It was

observed for the culture medium LB (control) and bacterial culture broths and their supernatants, a decrease of *E. fetida* survival, reaching 0% at 48 h for the non-diluted suspensions (10^0) and after 48 h for the 5.10^{-1}-diluted suspensions. The percentage of *E. fetida* survival recorded at 24 h for the 10^0 and 5.10^{-1}-diluted suspensions, respectively, was different ($p < 0.05$) between LB (79.99 and 100%) and culture broths of *E. cloacae* (11.11%) and *L. monocytogenes* (11.11 and 77.77%) but no difference ($p < 0.05$) was observed with *E. coli* (44.44 and 88.88%), *S. typhi* (66.66 and 100%) and *S. aureus* (44.44 and 100%). Concerning the bacterial supernatants, difference ($p < 0.05$) compared to the control LB was observed only with *E. cloacae* (11.11 and 77.77%). At 48 h, the percentage of *E. fetida* survival was no significantly different between LB and each of the tested bacteria for all dilutions at both broth and supernatant state. Overall, 40% of tested culture broths exhibit an effect on *E. fetida* compared to 20% of supernatants.

The observation about *G. candidum* culture broth may be the fact that this micro-organism is not pathogen for earthworms. Also, its belonging to the fungal reign might make it an appreciated food for *E. fetida.* Indeed, various species of earthworms have their feeding preferences towards fungi (Bonkowski et al., 2000). For example, according to Zirbes et al. (2012), the earthworm *Lumbricus terrestris* exhibits a preference for food substrates colonized by soil fungi *Mucor hiemalis* and *Penicillium* sp*.* The no observed effect of *G. candidum* culture broth on *E. fetida* in our study may suggest that earthworms are likely not stressed by the presence of fungi in their living environment. Earthworms due to their olfactory are rather attracted by fungi which synthesize chemical signals or volatile compounds, and feed them (Zirbes et al., 2011). So, the feeding mode of earthworms related to fungi could be used for biocontrolling the pathogen fungi in soil.

In contrast to culture broths of *E. cloacae* and *L. monocytogenes*, those of *E. coli*, *S. typhi* and *S. aureus* do not cause *E. fetida* mortality due to earthworm's antibacterial activity and immune system which may be discriminating. Indeed, to protect themselves or to mount their attack against soil organisms, earthworms produce the lysenin, a pore-forming toxin. Lysenin derived from coelomic fluid of *E. fetida* are particulary adapted to form pores in sphingomyelin-containing membrane (Sukumwang and Umezawa, 2013; Iacovache et al., 2008). Wang et al. (2006) reported that earthworms are infected by few micro-organisms although they live in an environment flocked with pathogens. The antibacterial barriers mainly include body wall, alimentary canal and parietal mucus.

After bacterial infection, lysozyme and antibacterial proteins (accounting for ones of responses of earthworms defense system) are enhanced and peaking at 4 h and 3 days, respectively (Hirigoyenberry et al., 1990). Besides, the *E. coli* strain used in this study (*E. coli* UCMA 10579 also called *E. coli* DH5α) is designed for laboratory use and is not a pathogen micro-organism (Chart et al., 2000; Jung et al., 2010). Therefore, one may consider *E. coli*, *S. typhi* and *S. aureus* as food for *E. fetida*. The results about culture broths of *E. coli* and *S. typhi* match with those of Eastman et *al.* showing that *E. fetida* eliminates human pathogens in domestic wastewater residuals (biosolids). There were for fecal coliforms and *Salmonella spp.* a 6.4-log and 8.6-log reduction in test samples (with earthworms) compared to the control (1.6-log and 4.9-log reduction), respectively (Eastman et al., 2001). Murry and Hinckley (1992) indicated the percentage decrease in the concentration of *Salmonella enteridis* cultured during 48 h in horse manure in presence of earthworms *E. fetida* compared to cultures without earthworms (8% versus 2%). In contrast with certain works about interactions *E. fetida* / *Enterobacteriaceae* showing *Enterobacter spp.* inhibited by earthworm's antibacterial activity or its digestive processes (Parthasarathi et al., 2007; Arslan-Aydoğdu and Çotuk, 2008), the *E. cloacae* strain used in this study reduces significantly *E. fetida* survival at both the culture broth level and the supernatant compared to control. This may be due to the antibacterial resistance and opportunist pathogen characteristics of this bacterium. In fact, *E. cloacae* is known as highly versatile and is capable of overproducing many antibiotic resistances such as AmpC β-lactamases, cephalosporinase that are able to render ineffective almost all antibiotic families (Davin-Reglis and Pages, 2015; Guérin, 2015). It is also able to form biofilm and to secrete various metabolites including cytotoxins (enterotoxins, hemolysins, pore-forming toxins) (Mezzatesta et al., 2012). Like *E. cloacae, L. monocytogenes* possesses enzymatic equipment that might inhibit the antibacterial activity of *E. fetida* and induce its pathogenicity.

Washed cells generate no mortality of *E. fetida* related to washing. Indeed, the washing of micro-organisms leading to washed cells (micro-organisms free from culture medium and metabolites) creates new conditions where these microbial cells or washed cells cannot properly express their virulence if needed. Then when possible, the stresses (washed cells) they cause are mild or moderate so that earthworms recover their steady state. By analogy to microbial stress during processing, Lado and Yousef (2002) reported that sub-lethal stress induces the expression of cell repair systems. These different effects (mortality or not) of tested micro-organisms under various status (culture broth, washed cell and supernatant) show that the filter paper contact toxicity test from OECD (1984) designed for early assessing the toxicity of chemicals in soil might be applied to micro-organisms. This constitutes a pilot study using the described OECD method for assessing the effect of micro-organisms on *E. fetida* and gives an overview of interactions earthworms / micro-organisms

occurring in soil.

Conclusion

All these tested pesticides formulations (Round-up GT Plus, Stratos Ultra Jardin, Polyflor, Capiscol, Polyvalent and KB Limace) do not lead to *E. fetida* mortality when they are used at their recommended agricultural concentrations. At this concentration, they generate mild or moderate stress on *E. fetida*. Based on the LC_{50} values, the toxicological profile of these pesticide formulations used on *E. fetida* is the following one. Capiscol (76.3 µg/cm^2) is classified as a very toxic chemical, Stratos Ultra Jardin (213.64 µg/cm^2), Polyvalent (305.2 µg/cm^2), Round-up GT Plus (457.8 µg/cm^2) and Polyflor (824.04 µg/cm^2) are considered as moderately toxic chemicals and KB Limace (2828.36 µg/cm^2) is in the range of relatively nontoxic chemicals. Among all tested micro-organism suspensions, earthworms *E. fetida* were stressed by *E. cloacae* UCMA 10580 (culture broth) and *L. monocytogenes* UCMA 6115 (culture broth and supernatant). Furthermore, the filter paper contact test OECD might be used as a tool to evaluate the response of *E. fetida* to abiotic and biotic stresses.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Economic viability of the canola crop with nitrogen applied in coverage in no-tillage system in Corbélia-PR, Brazil

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It is essential for the farmer, to have information of cultural costs when deploying a new crop or new technology. The cost of the study allows decision making for new technologies and use of viable inputs in production (Richetti, 2011; Souza et al., 2012). Zimmermann (2005), when studying technical and economic viability of canola crops, stated that canola is economically viable. The aim of this work was to assess the economic viability of different quantities of nitrogenous fertilizers applied in coverage of canola (Hyola 61) crop. The experiment was conducted in a eutrophic Red Latosol, with geographic location of 24°49'06" south latitude and 53°16'44" west longitude, altitude of 682 m and presents subtropical (Cfa) climate, in the Agrícola Andreis experimental farm, in Corbélia, Paraná. The experimental design consisted of randomized blocks with four replications and seven treatments, totaling 28 plots with an area of 31.5 m^2 each. Quantities used to verify canola response were: T1 to 0 kg ha^{-1} N (control); T2 to 25 kg ha^{-1} N; T3 to 50 kg ha^{-1} N; T4 to 75 kg ha^{-1} N; T5 to 25 kg ha^{-1} N + 27 kg ha^{-1} S; T6 to 50 kg ha^{-1} N + 54 kg ha^{-1} S (both in solid form) and T7 to Micro Xisto HF (liquid form) foliar fertilizer. An F-test (Analysis of Variance) was used in order to verify statistical difference among treatments, followed by Tukey's means comparison test, at 5% significance. Grain yield presented a statistically non-significant tendency to increase. Nitrogen top dressing did not provide economic return for the climatic conditions observed in this harvest.

Key words: *Brassica napus* L. var. oleifera, fertilizer, production.

INTRODUCTION

The first canola crops were cultivated in 1974 by Cooperativa Regional Tritícola Serrana Ltda – Cotrijuí, in Rio Grande do Sul, as an alternative for fallow lands and in rotation with wheat crops during winter. In Paraná, crops started being cultivated in the beginning of 1980, whereas expansion of cropped areas only happened after 2001. According to the Brazilian Department of Agriculture and Supply (Seab, 2012), in 2011/2012, Paraná harvested

12,454 hectares of canola, amounting to a grain yield of 20,683 tons and average yield of 1,661 kg ha^{-1}.

Canola cultivation is a viable economical alternative for winter crops in rotation with wheat and winter maize, besides being an option for agro-energetic purposes (Tomm 2006). It is important to mention that crop rotation systems maximize sustainability and decrease phytosanitary issues that lower yield and increase production costs on commercial crops due to diversity of crops of different plant species. Another factor that might influence in canola crop yield is the availability of nutrients in the soil, mainly nitrogen and sulfur. Normally, there is a deficiency of these two compounds in acid soils with low levels of organic matter, as stated by Tomm (2007). Nitrogen is the most requested nutrient and the one that most influences crop production whenever the remaining nutrients are found in satisfactory levels (Freitas et al., 2010; Melo et al., 2011).

Rheinheimer et al. (2007) observed that the areas with the largest canola crops in Brazil presented a deficiency of sulfur, mainly due to intense use of concentrated fertilizers without sulfur in their formulation and continuous extraction caused by harvesting. The reduction in organic matter quantity due to lack of crop rotation and increase of mineralization is another factor that contributes to sulfur decrease in the soil. Even with the incentive given by the state of Paraná to canola cultivation, it is important to assess the economic viability of this oilseed when setting up a new business. In the opinion of Richetti (2011) and Souza et al. (2012) the study of costs for implementing a crop aims to assist decision making, as well as adopting technologies and using inputs to obtain the best results in agricultural production.

Zimmermann (2005), when studying technical and economic viability of canola crops, stated that canola is economically viable and, in addition to being another option in winter crop rotation, it also makes possible to break pest and disease cycles. According to Souza et al. (2012), the gross revenue of a business is obtained by multiplying the total yield by the product unit value. Total business cost is given by the sum of all factors involving production cost, such as: applied agricultural inputs; performed agricultural operations; business administration; depreciation of improvements, machinery and equipments; cost, capital and land remuneration. Net earnings correspond to the difference between gross revenue and total cost. The business is only economically viable when the return is positive. This work aimed to assess productivity behavior and production cost of a canola crop under no tillage in function of nitrogen and sulfur top treatments.

MATERIALS AND METHODS

The experiment was carried out at Agrícola Andreis experimental farm in Corbélia, Paraná, in a typical Eutrophic Oxisol according to the Brazilian System of Soil Classification SiBCS (2009), with clay content superior to 65% according to laboratorial analyzes from Solanálise (2010). The farm is located at south latitude 24°49'06" and west longitude 53°16'44", altitude of 682 m and presents subtropical (Cfa) climate, as specified by Köppen's classification Iapar (2012).

The seeder/fertilizer set was used in the experiment, model PST3, with 7 rows, intra-row spacing of 45 cm and 10 m of length, loaded with canola Hybrid Hyola 61, covering an area of 31.5 m^2 for each plot of the treatment, with two border lines, as suggested by Tomm (2007) for the no tillage. The dose of 280 kg ka^{-1} of 10-18-18 NPK fertilizer, corresponding to 28 kg ha^{-1} N, 50 kg ha^{-1} P_2O_5 and 50 kg ha^{-1} K_2O, was used in the base fertilization. The sowing depths used consisted of 2 to 3 cm, with density of 25 seeds per meter, to provide a final density of at least 40 plants per square meter (Tomm et al., 2009).

Nitrogen and sulfur were top dressed manually 5 cm away from the canola row and foliar fertilizer application was done with an electric knapsack sprayer with 80 L ha^{-1} mixture, 45 days after emergence with damp soil (Tomm et al., 2009). The experiment design consisted of randomized blocks with four replications and seven treatments, totaling 28 plots, with an area of 31.5 m^2 each (Gomes, 1987). Treatments were conducted with seven different quantities of nitrogen and sulfur: T1: 0 kg ha^{-1} N (control); T2: 25 kg ha^{-1} N; T3: 50 kg ha^{-1} N, T4: 75 kg ha^{-1} N; T5: 25 kg ha^{-1} N + 27 kg ha^{-1} S; T6: 50 kg ha^{-1} N + 54 kg ha^{-1} S (both in solid form) and T7: 0.45 L ha^{-1} N + 0.1L ha^{-1} S (liquid form). The commercial fertilizers employed were: CO $(NH_2)_2$ (urea), as source of N; $(NH_4)_2$ $S0_4$ (ammonium sulfate), as source of N + S, and Micro Xisto HF foliar fertilizer, as source of N + S.

To keep the standard of the desired plants, pesticides had to be used to control pests. Therefore, *Diabrotica speciosa* (Germar) and *Elasmopalpus lignosellus* (Zeller) were controlled by means of spraying with a sprayer bar, employing pesticides Novalurom 15 g i.a ha^{-1} + Esfenvalerate 10 g i.a ha^{-1} with a mixture of 130 L ha^{-1} at the beginning of pest attack, 11 days after canola emergence (Domiciano and Santos, 1996; Zimmermann, 2005; Tomm et al., 2009).

The assessment of productivity was given by the total manual harvest of each treatment sample and converted into kg ha^{-1} according to Krüger et al. (2011). The economic return of each treatment was achieved by verifying variable costs (employed inputs and labor) added to the capital cost (depreciation of improvements, machinery, equipment and land remuneration), subtracted from the gross revenue, which is obtained by means of grain revenue (Carvalho 2011; Souza et al. 2012). An F-test (Analysis of Variance) was used in order to verify statistical difference among treatments, followed by Tukey's means comparison test, both at 5% significance (Gomes, 1987). Model presumptions were verified by applying Hartley's Fmax test for homogeneity of variances and Shapiro-Wilk's test for normality. Software ASSISTAT 7.6 beta was used for data analysis (Silva and Azevedo, 2009). Production variables as well as costs generated by

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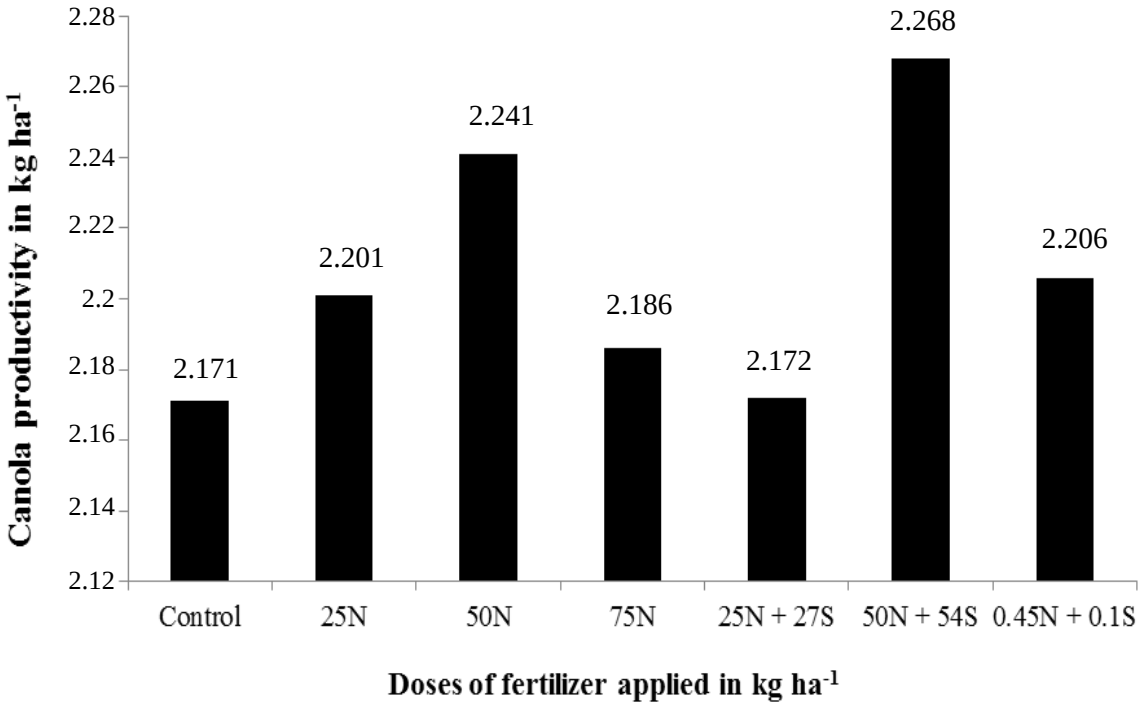

Figure 1. Canola production in kg ha^{-1} as a function of the different amounts of fertilizer applied.

each treatment were considered when determining the most indicated and economically viable treatment to obtain higher yield in oil production.

RESULTS AND DISCUSSION

Figure 1 shows the average values in canola productivity in kg ha^{-1} as a function of nitrogen (N) and sulfur (S) doses as top treatments (control = 0 kg ha^{-1} N; 25 N = 25 kg ha^{-1} N; 50 N = 50 kg ha^{-1} N; 75 N = 75 kg ha^{-1} N; 25 N + 27 S = 25 kg ha^{-1} N+ 27 kg ha^{-1} S; 50 N + 54 S = 50 kg ha^{-1} N + 54 kg ha^{-1} S and 0.45 N + 0.1 S = 0.45 L ha^{-1} N + 0.1 L ha^{-1} S (foliar fertilizer). Canola productivity in kg ha^{-1} as a function of the doses of nitrogen applied as top dressings in the treatment of 50 kg ha^{-1} N presented an increase of 70 kg ha^{-1} in comparison to the control, whereas, in the treatment with 75 kg ha^{-1} the yield increase was only 15 kg ha^{-1}. As for treatments with nitrogen + sulfur, there was a grain yield increase of 97 kg ha^{-1} in the treatment with 50 N + 54 S kg ha^{-1} in comparison to the control. The treatment with liquid nitrogen showed a yield increase of 35 kg ha^{-1} when compared to the control. Even though all treatments presented an increase in canola grain yield, such productivity was not statistically significant at a level of 5%.

According to Osório Filho et al. (2007), the lack of response to the sulfur added to the soil, may be related to the intake of atmospheric sulfur by rainwater, even in demanding crops. Jackson (2000) obtained results that differed from the ones found in this study in a research on canola productivity with five different experimental conditions and observed spring canola responses to different amounts of nitrogen and sulfur. In a study with nitrogen doses ranging from 50 to 200 kg ha^{-1}, Öztürk (2010) obtained a 47% increase in grain yield with a treatment that received 150 kg ha^{-1} N.

Borsoi et al. (2010) studied the effect of nitrogen and sulfur on Hybrid Hyola 43 and obtained statistically significant differences compared to the control with treatments with 38 kg ha^{-1} (urea) and 17 N + 18 S kg ha^{-1} (ammonium sulfate). The treatment with sulfur + nitrogen increased the yield in 20.9%. Karamanos et al. (2007) obtained an increase of 23.7% in canola yield with the use of nitrogen and sulfur in soils lacking these nutrients. Gao et al. (2010) in a study on canola yield with the application of 84 and 168 kg ha^{-1} N in two locations did not obtain any increase in grain yield. The same was observed by Rigon et al. (2010) when assessing canola response to sulfur and nitrogen applied to the cover in plots. The behavior of fenometric variables depending on fertilization of nitrogen applied to the coverage (Figure 2) shows regression curves obtained for the average values of the number of siliquas per plant, mass of a thousand grains, Canola production in kg ha^{-1}, and Canola oil content. It can be seen in Figure 2a that the number of

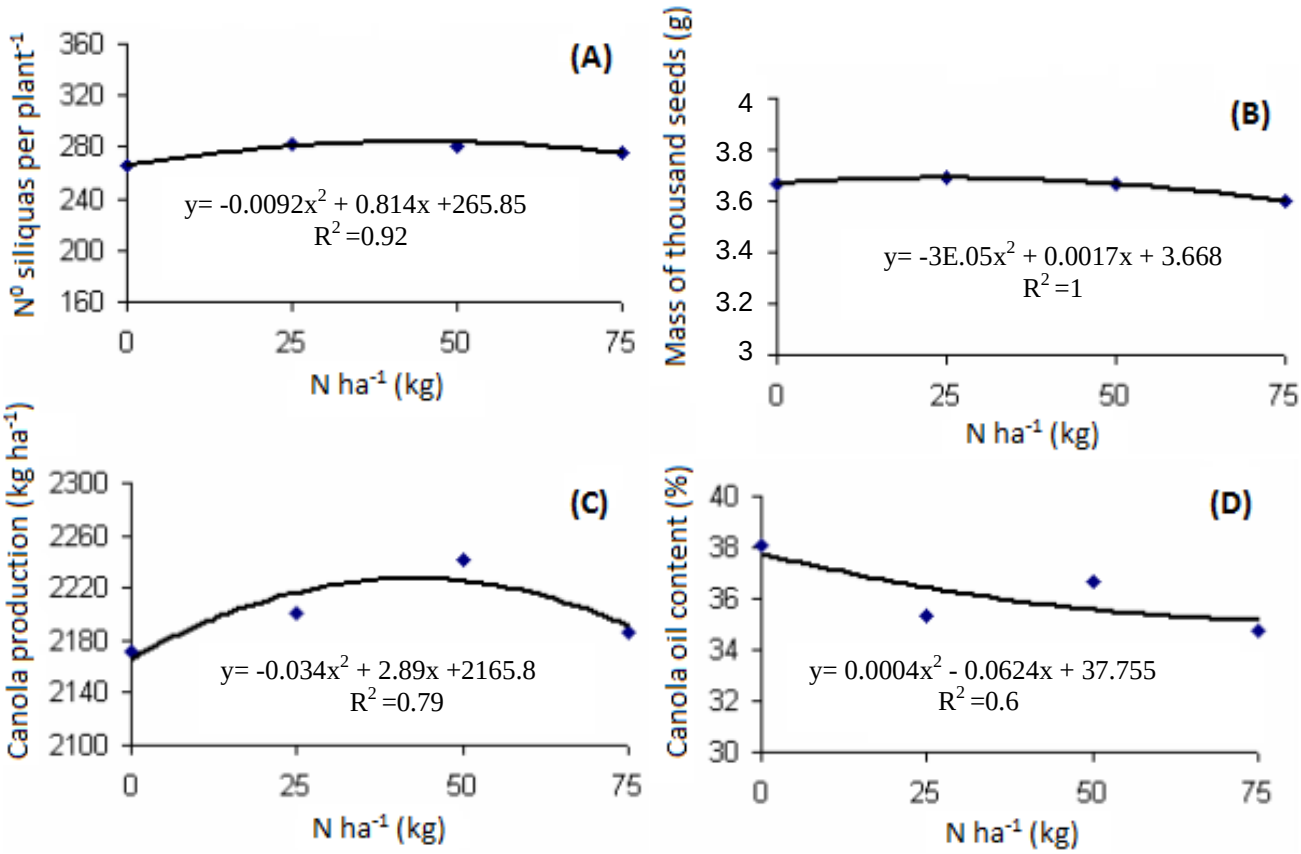

Figure 2. Curves obtained for average values of number of siliquas per plant (A), mass of a thousand seeds (B), Canola production in kg ha^{-1} (C), and Canola oil content (D) according to the fertilization of N applied to the coverage.

Table 1. Canola economic return according to the amounts of nitrogen and sulfur applied as top treatments in one hectare in 2011.

Variables	T1	T2	T3	T4	T5	T6	T7
		----------R$ ha^{-1}----------					
Gross revenue	1,519.76	1,540.94	1,569.33	1,530.36	1,520.18	1,587.63	1,544.77
Total cost	890.82	977.37	1,048.92	1,120.47	1,005.11	1,104.39	932.40
Net earnings	628.94	563.57	520.41	409.89	515.07	483.24	612.37

T1 = 0 kg ha^{-1} N (control); T2 = 25 kg ha^{-1} N; T3 = 50 kg ha^{-1} N; T4 = 75 kg ha^{-1} N; T5 = 25 kg ha^{-1} N + 27 kg ha^{-1} S; T6 = 50 kg ha^{-1} N + 54 kg ha^{-1} S and T7 = 0.45 L ha^{-1} N + 0.1 L ha^{-1} S (foliar fertilizer).

siliquas per plant follows a quadratic relationship, reaching the maximum point between 25 and 50 kg of N ha^{-1} (in the amount of 44.24 kg N ha^{-1}). The Figure 2b presents the regression curve for the mass of thousand grains according to the doses of nitrogen applied coverage; it is observed that the maximum mass point is obtained between 25 and 50 kg N ha^{-1} (in the value 28.33 kg of N ha^{-1}). In Figure 2c, the productivity kg ha-1 follows a quadratic relation, reaching the maximum point between 25 and 50 kg N ha^{-1} (in the value 42.50 kg of N ha^{-1}). It is observed that the grains in oil content in Figure 2d, decreases as the coverage fertilization with nitrogen increases. Similar results were obtained by Ahmad et al. (2007) when studying Canola´s response to nitrogen fertilization. Jackson (2000) stated that canola decreases seed oil content when larger quantities of nitrogen are applied, possibly due to the delay in the crop´s maturation. Another probable cause of this reduction in oil content is the fact that such nutrient is one of the main components of proteins that leads to an increase in

Table 2. Canola total cost (R$ ha^{-1}) and relative cost in one hectare according to the amounts of nitrogen and sulfur applied to the cover in 2011.

Treatments	Variables	
Kg (ha^{-1}) of N and S	Total cost in R$ (ha^{-1})	Relative cost
T1	890.82	100
T2	977.37	109.7
T3	1,048.92	117.7
T4	1,120.47	125.8
T5	1,005.11	112.8
T6	1,104.39	124.0
T7	932.40	104.7

T1 = 0 kg ha^{-1} N (control); T2 = 25 kg ha^{-1} N; T3 = 50 kg ha^{-1} N; T4 = 75 kg ha^{-1} N; T5 = 25 kg ha^{-1} N + 27 kg ha^{-1} S; T6 = 50 kg ha^{-1} N + 54 kg ha^{-1} S and T7 = 0.45 L ha^{-1} N + 0.1 L ha^{-1} S (foliar fertilizer).

protein percentage and a decrease in oil content (Öztürk, 2010). Table 1 shows the economic return of the canola crop according to the amounts of nitrogen and sulfur applied as top treatments.

The experiment showed that the highest net earnings were obtained with T1, which did not receive any nitrogen or sulfur to its cover, whereas, the lowest net earnings were verified in the treatment that received 75 kg ha^{-1} nitrogen to its cover. Such results are similar to those obtained by Souza et al. (2012), in a study on maize response to nitrogen and sulfur fertilization, this was applied as top treatments in doses of 100, 150 and 200 kg ha^{-1}, in harvests 2008 and 2009. Canola production cost was higher with the addition of nitrogen sources applied to the cover, which ranged from R$ 890.82 to R$ 1,120.47 ha^{-1} for treatments without N and with 75 kg ha^{-1} N, respectively (Table 1). Relative costs in one canola hectare, according to the applications of nitrogen and sulfur to the cover, are described in Table 2. The treatment which received 75 kg ha^{-1} N had an increase of 25.8% in production cost when compared to the control treatment, which did not receive N to its cover. The production cost with applications of 50 N + 54 S kg ha^{-1} was 1.8% lower with the application of 75 kg ha^{-1} N to the cover. The treatment that received foliar fertilizer application had the lowest relative cost increase in relation to the control treatment. The participation of variables that constitute the production cost of a canola hectare is shown in Table 3.

The total canola cost in one hectare was 21.21 sacks for the treatment without nitrogen as top dressing and 26.68 sacks for the treatment that received 75 kg ha^{-1} N. For this treatment, the cost increase was 5.47 sacks ha^{-1} and the canola production increase was 15 kg ha^{-1} (0.25 sacks ha^{-1}). The treatment with 50 kg ha^{-1} N provided an increase of 70 kg ha^{-1} (1.17 sacks) in production and the cost increase was 3.76 canola sacks ha^{-1}. The treatment with 50 N + 54 S kg ha^{-1} presented a production increase of 97 kg ha^{-1} (1.62 sacks) and the cost increase was 5.08 canola sacks ha^{-1}. The treatment with foliar fertilizer had a cost increase of 2.06 sacks ha^{-1} and a canola production increase of 35 kg ha^{-1} (0.58 sacks). The cost with nitrogen fertilizers applied to the cover was higher than the addition to the canola production cost in all treatments. By considering the participation of nitrogen fertilizers applied as top treatments in one hectare in the total canola cost, one can verify that the variation ranges from 0% (for the treatment that did not receive cover fertilization) to 19.19% (for the treatment that received 75 kg ha^{-1} de N).

Conclusion

Canola productivity did not present significant statistical difference in all treatments with cover fertilization. The gross income obtained with canola decreased with the applications of nitrogen to the cover. Nitrogen fertilization applied as top dressing did not provide economic return in this harvest under the climatic conditions observed. It concludes also that it is important to perform technology diffusion activities to increase canola yield. The environmental education, agricultural and vocational education for rethinking management proposals is for new farmers of canola cultivation. The internationalization of small and medium enterprises (SMEs) is towards canola exporting at a wider marketplace. The social acceptability or competitive land use of other prosperous and prolific cultivation species, were applicable. The governmental policies and political initiatives helped in strengthening the perspectives of the canola future.

Conflict of interest

The authors have not declared any conflict of interest.

Table 3. Variables of production cost of one hectare of canola under no tillage in the city of Corbélia, Paraná, harvest 2011 for family farming.

Cost components	T1	T2	T3	T4	T5	T6	T7
				-----------%-----------			
1. Inputs							
Canola seeds	12.80	11.66	10.87	10.17	11.34	10.32	12.23
Maintenance fertilizer	40.92	37.30	34.76	32.54	36.27	33.01	39.1
Herbicides	1.71	1.56	1.45	1.36	1.51	1.38	1.63
Cover fertilizer	0.00	7.32	13.64	19.16	9.89	17.98	3.22
Insecticides	2.05	1.87	1.74	1.63	1.82	1.65	1.96
2. Agricultural operations							
Sowing	4.37	3.98	3.71	3.47	3.87	3.52	4.17
Herbicide application	1.30	1.19	1.10	1.03	1.15	1.05	1.24
Insecticide application	1.30	1.19	1.10	1.03	1.15	1.05	1.24
Fertilizer application	0.00	1.54	1.43	1.34	1.49	1.36	1.24
Mechanical harvest	10.10	9.21	8.58	8.03	8.95	8.15	9.65
3. Other costs							
Manpower	4.58	4.17	3.89	3.64	4.06	3.69	4.38
Technical assistance	1.75	1.59	1.49	1.39	1.55	1.41	1.67
Agricultural insurance (Proagro)	1.75	1.59	1.49	1.39	1.55	1.41	1.67
4. Depreciations							
Improvement depreciation	2.37	2.16	2.01	1.88	2.10	1.91	2.26
Machinery depreciation	7.10	6.47	6.03	5.65	6.30	5.73	6.79
Equipment depreciation	4.99	4.55	4.24	3.97	4.42	4.03	4.77
5. Remuneration of factors							
Land remuneration (3% land value)	2.91	2.65	2.47	2.32	2.58	2.35	2.78
Total	100	100	100	100	100	100	100
		----------Sacks (ha^{-1})----------					
Cost in 60-kg sacks	21.21	23.27	24.97	26.68	23.93	26.29	22.20

T1 = 0 kg ha^{-1} N (control); T2 = 25 kg ha^{-1} N; T3 = 50 kg ha^{-1} N; T4 = 75 kg ha^{-1} N; T5 = 25 kg ha^{-1} N + 27 kg ha^{-1} S; T6 = 50 kg ha^{-1} N + 54 kg ha^{-1} S and T7 = 0.45 L ha^{-1} N + 0.1 L ha^{-1} S (foliar fertilizer).

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Supplemental value of leaf based concentrates with *Panicum maximum* hay on performance of West African dwarf goats

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A study was conducted to evaluate the effects of supplementation of a leaf based concentrate consisting of a 1:1 leaf mixture of *Vernonia amygdalina* and *Tithonia diversifolia* (VATD) as a direct replacement for brewers dried grains (BDG) on feed intake, growth performance and digestibility by West African Dwarf (WAD) goats on a basal diet of *Panicum maximum* hay. Twenty five growing WAD goats weighing between 8.07 - 9.60 kg were allotted into five dietary treatment groups in a completely randomized design with five goats per treatment. The mixed leaf meal (VATD) were included in the diets at 0% (T1), 5% (T2), 10% (T3), 15% (T5), and 20% (T5) of the total diet. The experiment lasted for twelve weeks. Results showed that dry matter (DM) intake (g/kg $W^{0.75}$/day) was lowest (P<0.05) in control diet T1 (72.02), and higher in T3 (92.78) than T2, T4 and T5 (88.69, 86.61 and 86.70, respectively). Crude protein (CP) intake (g/kg $W^{0.75}$/day) peaked at T3 and progressively reduced thereafter in T4 (15.10), T5 (14.83) and T2 (14.16). CP intake was higher (P<0.05) in T3 than T1. Daily weight gain (g/day) of goats ranged from 32.97 (T4 and T5) to 38.40 (T3). Crude protein digestibility was higher (P<0.05) in T5 (89.49%) than T1 (84.23%) and T4 (84.19%) but similar in T2 (85.47%) and T3 (86.10%). Better nitrogen intake (15.10 g/day) was observed in goats fed T4 compared to those fed T1 (14.32) and T2 (14.16). Rumen pH values were higher in T1 than T2, T3, T4 and T5. Ammonia N content (mg/100ml) of the rumen was similar in goats fed T2 (9.92), T3 (10.07) and T5 (9.80) but higher (P<0.05) than those fed T1 (8.19). The result suggests that WAD goats fed VATD leaf meal based concentrates perform better on diets with levels of mixed concentrates not exceeding 10%.

Key words: Growth, Leaf meal, nitrogen utilization, *Tithonia diversifolia, Vernonia amygdalina*, WAD goats.

INTRODUCTION

Utilizing the vast amount of grasslands in Nigeria remains one of the most economical ways to achieve growth in ruminant production. However, these grasses and pastures are deficient in nitrogen and digestible nutrients

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for optimum fermentative rumen digestion (VanSoest, 1982). Consequently, this results in low intake which can be improved by tree fodder supplementation (Topps, 1992; Abdulrazak et al., 2000). According to Kanjanapruthipong and Leng (1998), there was an improvement in the performance of rams fed poor quality grass diets supplemented with *Gliricidia maculata* at an optimum level of inclusion of 20 - 25%. Similarly, Tona et al. (2014) reported improved intake and live weight gains when they fed concentrate diets with graded levels of *Moringa oleifera* leaves at 15% level of inclusion to goats. Tree browses have tremendous potential as supplements to the low quality forages or grass diets fed to goats. Most tree browses, however, contain tannins that may adversely affect their utilization (Makkar et al., 1989; Makkar, 1993). It is therefore important to process forages to remove the anti-nutrients. Different processing methods used to remove anti-nutrients from forages include wilting, air drying, sun drying, and oven drying. Where tannin contents have been reduced through processing to a tolerable level, the tannin may proffer a by-pass characteristic to the protein, hence making it utilizable by the animal (Aganga and Tshwenyane, 2003; Barry and McNabb, 1999).

Wild sunflower (*Tithonia diversifolia)* is one of the new foliages, which is considered by many to be a valuable green manure. In Western Kenya, it is renowned as a component of agro-forestry systems as it is rich in N, P and K, which are essential for soil fertility. According to the reports of Mahecha and Rosales (1996), the crude protein content in the foliage of *T. diversifolia* was 24.2% on DM basis and about 40% of the protein was soluble. The percentage of NDF (on DM) was 35.3 and 30.4% ADF. The rumen degradable protein (*in sacco*) was about 90% in 48 h (Rosales, 1996). The low content of tannins reported for *Tithonia* foliage (Wambui et al., 2006) supports the idea that the protein may well be highly soluble with poor "bypass" characteristics (Preston and Leng, 1987). The fact that loss of nitrogen in the urine was higher for Tithonia than for Calliandra or Sesbania (Wambui et al., 2006) supports this suggestion and further reveals that rumen ammonia levels are high in *Tithonia*, resulting in high excretory levels of urea in the urine. A high proportion of N released in the rumen could contribute to higher N retention if it is fed in combination with high tannin foliage in the diet (Barry, 1987).

Bitter leaf (*Vernonia amygdalina*) is a shrub with petiole leaf of about 6mm diameter, green with a characteristic odour and bitter taste. It is propagated vegetatively while growing under a range of ecological zones in Africa and produces large mass of forage and is drought tolerant (Bonsi et al., 1995). Although the leaves are used for human consumption, its production outweighs its human consumption suggesting that the excess may be utilized as feed for ruminants without detrimental effects on general productivity of animals. Presence of tannins was reported in the forages of Bitter leaf (Daodu and Babayemi, 2009). This situation suggests that the foliage of *V. amygdalina* could confer bypass properties on the ruminal protein derived from *T. diversifolia* leaves.

The objective of this study was to examine the effects of supplementation of concentrates containing 1:1 leaf mixture of *V. amygdalina* and *T. diversifolia* on voluntary feed intake, diet digestibility and growth performance for growing West African Dwarf goats fed a basal diet of *Panicum maximum* hay.

MATERIALS AND METHODS

Study site and animal management

This study was conducted at the Goat Unit, Teaching and Research Farm, Institute of Agricultural Research and Training, Moor Plantation, Ibadan, Nigeria. Twenty-five (25) female growing West African Dwarf (WAD) goats of approximately 6-7 months old and weighing between 8.50 – 9.60 kg were used for the study. The goats were housed intensively in well ventilated individual pens in an open sided housing unit, which had previously been cleaned and disinfected with morigard solution before the arrival of the animals. They were administered with *Pestes des Petit* Ruminants vaccine, broad spectrum antibiotics (Kepro-oxytet 20% LA injection) and ivomec® to eliminate possible ecto- and endoparasites. The floor of each pen was covered with wood shavings as bedding material. Animals were allowed a 2-week adaptation period during which they were introduced to free feeding of compounded concentrates and *P. maximum* forages. At the end of the adaptation period, animals were randomly divided into five dietary treatments of five animals per treatment balanced for weight.

Forage collection and feed preparation

T. diversifolia and *V. amygdalina* leaves were harvested, air dried under shade, and then ground in a hammer mill. Five experimental diets were formulated and designated: T1 (0% *V. amygdalina and T. diversifolia)*, T2 (5% *V. amygdalina and T. diversifolia)*, T3 (10% *V. amygdalina and T. diversifolia)*, T4 (15% *V. amygdalina and T. diversifolia),* and T5 (20% *V. amygdalina and T. diversifolia)*. The dietary treatments consisted of different inclusion levels of ground 1:1 mixture of *V. amygdalina* (VA) and *T. diversifolia* (TD) leaf meal in a compounded ration (Table 1).

Feeding trial

Concentrate diets were fed in the morning (08.00 h) and *P. maximum* hay in the afternoon (14.00 h). Fresh water was provided *ad libitum*. Block of saltlick was permanently placed in each pen. During the first two weeks, animals were served 200 g of concentrate diet and 300 g of *P. maximum* hay allowing a proportional refusal of 10% of total daily amount offered (Ondiek et al., 2010). Refusals were collected daily, weighed and recorded. Animals were weighed before the commencement of the experiment and then weekly for 12 weeks. Each weighing was done after 10-12 h withdrawal of feed.

Digestibility study

Digestibility trial was carried out by the total faecal and urine collection method of McDonald et al. (1995). Immediately after 12 weeks of the feeding trial, animals were weighed and each animal

Table 1. Chemical composition of *Panicum maximum*, *Vernonia amygdalina*, *Tithonia diversifolia* and diets fed to WAD goats (g/100 g DM).

Composition	*PM*	*VA*	*TD*	*VATD leaf meal*	Diets				
					T1	T2	T3	T4	T5
Dry matter	86.79	87.96	87.74	85.44	86.31	87.14	86.25	86.46	87.03
Crude protein	11.28	17.18	18.26	17.72	19.65	19.14	18.63	18.12	17.61
Ether extract	3.26	4.28	5.11	6.52	3.67	3.54	3.47	3.63	2.96
Ash	9.88	12.24	11.38	11.60	9.67	8.75	9.73	8.89	8.92
NDF	65.58	59.21	57.15	58.00	41.69	52.37	52.96	54.88	55.26
ADF	39.79	35.44	31.47	33.30	23.48	27.85	27.24	28.15	28.56
ADL	11.28	10.35	9.21	10.12	5.26	6.28	6.37	8.43	8.38
NFE	30.98	37.47	37.17	36.65	35.84	36.20	34.50	33.90	34.58

PM= *Panicum maximum*; VA= *Vernonia amygdalina*; TD= *Tithonia diversifolia*; VATD leaf meal= Mixture of *Vernonia amygdalina* and *Tithonia diversifolia* leaf meal; DM= Dry matter; NDF=neutral detergent fibre; ADF= acid detergent fibre; ADL= acid detergent lignin; NFE= nitrogen free extract; T1=0%VATD meal, T2=5%VATD meal, T3=10%VATD meal, T4=15%VATD meal, T5=20%VATD meal.

was penned in an individual cage for 14 days, with a 7 day adjustment and another 7 days collection period. Faeces and urine voided were collected. Individual total urine was collected and a 10% aliquot was kept in a refrigerator (0-4°C) for analysis. Faecal samples wrapped in aluminium foil were dried at 65^0C for 48 h, milled and stored in air-tight bottles until analyzed. During the last three days of the digestibility trial, approximately 10 ml of rumen liquor was collected from the goats before feed was offered and thereafter 2, 4, 6 and 8 h post feeding, using a stomach tube. The pH of the sample was determined immediately using an ionizable pH meter and the sample was thereafter strained through a double layer of clean cheesecloth. About 10 ml of the liquid fraction was sampled, acidified with 2 ml of 10% H_2SO_4 and stored in a refrigerator for analysis of NH_3-N (Han et al., 1989).

Chemical composition

Chemical composition (Crude protein, Ether extract, Ash and Dry matter) of *P. maximum* hay, *V. amygdalina*, *Tithonia diversifolia* and the experimental diets were determined according to the methods of AOAC (1990). Neutral detergent fibre (NDF), Acid detergent fibre and Lignin components of the diets were determined according to the procedure of VanSoest et al. (1991).

Statistical analysis

All data collected were subjected to analysis of variance (ANOVA) using the procedure of SAS (1999). Significant treatment mean values ($p<0.05$) were compared using the Duncan Multiple Range Test of the same package.

RESULTS AND DISCUSSION

The chemical composition (g/100 g DM) of *P. maximum*, *V. amygdalina*, *T. diversifolia* and compounded diets is shown in Table 1, while Table 2 shows the gross composition (g/100 g DM) of experimental diets. The results in Table 1 revealed that *P. maximum*, *V. amygdalina*, *T. diversifolia* and the different experimental concentrate diets had relatively high dry matter (DM) content. It ranged from 86.79 in *P. maximum* to 87.96 in *V. amygdalina*, while in the concentrate diets, it ranged from 86.25 in T3 to 87.14 in T2. Dry matter values for *P. maximum* and the browses in the current study are similar to values of 85.37 reported by Odedire and Oloidi (2014) for *T. diversifolia*, but higher than 78.44 reported for Guinea grass by Okoruwa et al. (2014). The differences in dry matter values may have been as a result of harvesting periods. The crude protein (CP) content of *Panicum maximum*, *V. amygdalina* and *T. diversifolia* ranged between 11.28 and 18.26 while CP for concentrate diets ranged between 17.61 and 19.65 g/100 g DM. Values for CP (21.14), NDF (63.20) and ADF (43.25) reported by Odedire and Oloidi (2014) for *T. diversifolia* were higher than values observed in this experiment. Genetic differences (variety), stage of maturity and location (Osuga et al., 2006) may have resulted in the differences in chemical composition. The CP content of *P. maximum* (11.28) fell within the range (105 to 133 g/kg DM) reported for tropical grasses (Topps and Oliver, 1993) and higher than 7.63 (Yousuf et al., 2007) for *P. maximum*. CP value for *P. maximum* (11.28 g/100 g DM) is higher than 7% indicated as the minimum for microbial growth and optimum roughage intake (Marschner, 1995; Minson, 1981). The high crude protein of the grass could be attributed to the young age of the grasses at the time of harvest (Most were about 4-6 weeks at the time of harvest). Marschner (1995) reported that at any nitrogen rate, the nitrogen concentration in emergent leaves and newly expanded leaf lamina was higher than in mature leaf lamina. Plants transfer nitrogen to younger tissues, which import higher amounts of nutrients than they export prior to reaching maturity. NDF content of diets increased (41.69 – 55.26 g/100 g) with a corresponding increase in the level of VATD meal it contained while the content of ADF also increased from 23.48 – 28.56 g/100 g in the concentrate diets. The increase in NDF and ADF as inclusion level of leaf meal

Table 2. Gross composition (g/100 g DM) of experimental diets.

Ingredients	Treatments				
	T1	T2	T3	T4	T5
Maize	20	20	20	20	20
BDG	30	25	20	15	10
VATD Meal	-	5	10	15	20
Wheat offal	15	15	15	15	15
GNC	5	5	5	5	5
PKC	25	25	25	25	25
Limestone	4.5	4.5	4.5	4.5	4.5
Salt	0.25	0.25	0.25	0.25	0.25
Grower Premix	0.25	0.25	0.25	0.25	0.25
Total	100	100	100	100	100
Calculated:					
% Crude Protein	19.65	19.14	18.63	18.12	17.61
TDN (%)	69.68	69.01	68.14	67.29	66.43

ME= Metabolizable energy; VATD= *Vernonia amygdalina* and *Tithonia diversifolia* leafmeal. T1=0%VATD meal, T2=5%VATD meal, T3=10%VATD meal, T4=15%VATD meal, T5=20%VATD meal.

Table 3. Performance characteristics, pH and rumen ammonia of WAD goats fed *Tithonia diversifolia* and *Vernonia amygdalina* leaf meal based concentrates.

Feed Intake g/d DM	Treatments						
	T1	T2	T3	T4	T5	P-value	SEM
Panicum maximum	346.72	361.92	342.68	354.39	357.72	0.0435	4.73
Conc. + Leafmeal	256.41^{b}	295.02^{a}	272.64^{ab}	270.30^{ab}	267.91^{ab}	0.0311	5.41
Total	603.13	656.94	615.32	624.69	625.63	0.0442	10.14
Dry matter intake (g/kg $W^{0.75}$/day)	77.02^{c}	88.69^{b}	92.78^{a}	86.61^{b}	86.70^{b}	0.0256	1.98
Crude protein intake (g/kg $W^{0.75}$/day)	13.20^{c}	14.16^{b}	16.07^{a}	15.10^{b}	14.83^{b}	0.0037	0.34
NDF intake (g/kg $W^{0.75}$/day)	49.30^{c}	61.76^{b}	64.31^{a}	61.35^{b}	61.45^{b}	0.0001	1.83
Initial weight (kg)	9.60	8.70	8.07	8.50	8.57	0.0483	1.55
Final weight (kg)	12.83^{a}	12.00^{ab}	11.33^{b}	11.50^{b}	11.57^{b}	0.0002	2.25
Weight gain (g/day)	35.49^{b}	36.26^{b}	38.40^{a}	32.97^{c}	32.97^{c}	0.0003	1.70
pH (%)	6.15^{a}	5.82^{b}	5.68^{b}	5.73^{b}	5.63^{b}	0.0020	0.13
Ammonia N (mg/100ml)	8.19^{c}	9.92^{b}	10.07^{b}	10.75^{a}	9.80^{b}	0.0001	0.27

^{abc}: means in the same row with different superscripts are significant (P<0.05), T1=0%VATD meal, T2=5%VATD meal,T3=10%VATD meal,T4=15%VATD meal,T5=20%VATD meal.

increased could be attributed to the high cell wall constituents usually present in leaf meals (Ambrasu et al., 2004). The higher CP value and lower NDF, ADF and ADL in both *V. amygdalina* and *T. diversifolia* compared to *P. maximum* presents the two forages as adequate in ruminant nutrition.

Table 3 shows the performance characteristics, pH and rumen ammonia of WAD goats fed *T. diversifolia* and *V. amygdalina* leaf based concentrates. Dry matter (DM) intake (g/kg $W^{0.75}$/day) was lowest (P<0.05) in control diet T1 (77.02), and higher in T3 (92.78) than T2, T4 and T5 (88.69, 86.61 and 86.70, respectively). This trend of DM intake revealed an initial increase in intake till it peaked at 10% VATD leaf meal inclusion level and then a decrease. This trend was consistent with the reports of Abdu et al. (2014) where DM intake of *Zizypus mauritiania* leaf meal based diet progressively increased till the 10% inclusion level and then decreased. However, the findings in this study does not support the reports of Ondiek et al. (2010) who reported a progressive increase in DM intake as leaf meal (*Maerua angolensis* and *Zizyphus mucronata*) inclusion in the diet increased. DMI values ranging from 54.60 to 59.60 g/$kg^{0.75}$ for WAD goats on groundnut hay basal diets, and offered moringa and bamboo foliages as supplements were reported by Asaolu et al. (2010). Higher intake in the leaf meal containing diets supports

the hypothesis that there are benefits in feeding mixtures of forages on DM intake and digestibility (Asaolu et al., 2012). The high intake associated with the 10% leaf meal diet suggests positive associative effect of the components of the leaf meal at that level of inclusion.

CP intake (g/kg $W^{0.75}$/day) was significantly ($P<0.05$) higher in T3 (16.07) than T1 (13.20), showing a peak at T3 and progressively reducing thereafter in T4 (15.10) and T5 (14.83). In an earlier study (Vranic et al., 2009), intake in ruminants was also influenced by a taste related factor-palatability. However, apart from nutritional composition, animals tend to consume more of palatable diet (Ibeawuchi et al., 2002). VATD leaf meal is not a very palatable feed ingredient due to its bitter taste. Increasing levels of VATD leaf meal mixtures in the diets may explain why the VATD leaf meal diets intake reduced after Treatment 3. The bitter taste, however, did not prevent animals consuming the leaf meal based diets to perform better than the control. Meanwhile, among the VATD leaf meal diets, Treatment 3 may have yielded the best-synchronized release of nitrogen and carbohydrate (Silva and Orskov, 1985) in the rumen required for microbial protein synthesis. This may have influenced the observed superior dry matter intake for goats on Treatment 3. NDF intake (g/kg $W^{0.75}$/day) was lowest ($P<0.05$) in T1 (49.30) and highest ($P<0.05$) in T3 (64.31). These values also exhibited the same trend as the DMI (Dry Matter Intake) and CPI (Crude Protein Intake) where increase in values terminated at Treatment 3 followed by a progressive decrease. The NDF intakes, when converted to g/day (361.92 – 397.75) were similar to NDF intake values of 325.9 - 347.10 reported by Oni et al. (2013) for WAD goats fed ensiled cassava leaves with or without molasses and caged layer waste. Weight gain (g/d) of goats was significantly affected by the treatment. Goats fed T3 (10%VATD meal) had significantly ($P<0.05$) higher weight gain (38.40 g) than those fed T1 (35.49 g), T4 (32.97 g) and T5 (32.97 g). This observation was expected since protein supplementation and intake is directly proportional to weight gain (Shahjalal et al., 1997). Earlier work (Negesse et al., 2001) observed increased Average Daily Gain in Saanen kids with the diet containing 17.6% CP when compared with 14.4, 11.4 and 8.7% CP levels. Likewise, (Shahjalal et al., 1997) reported that growth rate of grazing Black Bengal goats can improve under conditions of increased protein supplementation. Weight gain (g/day) of goats observed in this study (32.97 – 38.40) were higher than values (-4.91 – 17.4) reported by Ondiek et al. (2010) for growing small east African goats fed Rhodes grass hay supplemented by 1:1 mixture of *M. angolensis* and *Z. mucronata* leaf browses.

Rumen pH in all treatments was similar except for the control where it was higher. Rumen pH is an important factor that measures the alkalinity and acidity of rumen contents (Cabrita et al., 2006). Okoruwa et al. (2014) reported that for optimum rumen microbial fermentation, the rumen pH should lie between 6.00 and 6.80. However, all values reported for pH in this study fall below 6.00 except for the control (6.15) but this did not translate into reduction in weight gain as may be expected. Reduced rumen pH in the present study may be due mainly to reduced proteolysis, degradation of peptides and deamination of amino acids in the rumen. Reduction in pH between the control diet and other diets could probably be attributed to the crude protein contributed by the leaf meals in the concentrate diets since there was a gradual decrease in crude protein level in diets as more VATD leaf meal replaced brewers' dried grains.

Ammonia N content (mg/100 ml) of the rumen was similar in goats fed T2 (9.92), T3 (10.07) and T5 (9.80). Rumen NH_3-N values for goats in T4 (10.75) were higher ($P<0.05$) than those fed T1 (8.19). Ammonia N values (8.19 - 10.75 mg/100 ml) observed in this study compared favourably with values (8.99 – 12.70) reported by Ondiek et al. (2010), but lower than values reported for goats fed guinea grass and different levels of avocado seeds with orange peels (Okoruwa et al. (2014). The most suitable rumen NH_3- N concentration levels for microbial activities range between 5 and 20 mg/100 ml (Jyoti et al., 2000). This indicates that values observed for rumen NH_3- N in this study are adequate for microbial activities.

Apparent digestibility of diets by WAD goats fed *V. amygdalina* and *T. diversifolia* leaf meal based concentrates are shown in Table 4. Crude protein digestibility was higher ($P<0.05$) in T5 (89.49%) than T1 (84.23%). NDF digestibility was higher ($P<0.05$) in T5 (92.08) than other treatment diets, but similar in T2 (87.17), T3 (87.88) and T4 (88.99). All digestibility coefficients showed high digestibility levels which indicate that all diets were highly degraded in the rumen (Mapoon, 1980). Improvements in the digestibility values of DM and CP could be attributed to the positive influence of VATD leafmeal on the rumen environment in the goats. Forage mixtures used to replace brewers dried grains could have led to synchronized fermentability of individual chemical constituents leading to associative effects and improvements in DM intake and digestibility (Sinclair et al*.,* 1995; Rosales and Gill, 1997).

Nitrogen utilization of WAD goats fed *T. diversifolia* and *V. amygdalina* leaf based concentrates is shown in Table 5. Nitrogen intake (g/day) of goats fed T4 (15.10) was higher ($P<0.05$) than those fed T1 (14.32) and T2 (14.16) while goats fed T3 (14.76) and T5 (14.88) had similar values. This observation seems not to be consistent with the protein levels of the diet fed, since there was a gradual decrease in level of protein as VATD leaf meal increased in the diet. However this trend observed for nitrogen intake is consistent with protein intake for goats. Earlier study (Okoruwa et al., 2013) reported that crude protein combination in a diet has significant effect on the nitrogen intake of sheep.

Table 4. Apparent digestibilities (%) of diets by WAD goats fed *Vernonia amygdalina* and *Tithonia diversifolia* leaf meal based concentrates.

Parameters	Diets					P-value	SEM
	T1	T2	T3	T4	T5		
Dry matter	87.27^{b}	97.47^{a}	97.71^{a}	97.48^{a}	97.27^{a}	0.0219	0.15
Ether extract	92.25	92.51	92.63	93.16	94.74	0.8969	0.81
Crude protein	84.23^{b}	85.47^{ab}	86.10^{ab}	84.19^{b}	89.49^{a}	0.0096	0.52
NDF	76.33^{c}	87.17^{b}	87.88^{b}	88.99^{b}	92.08^{a}	0.0003	0.41
ADF	78.11^{c}	89.01^{b}	90.41^{ab}	90.10^{ab}	94.72^{a}	0.0001	0.35

^{abc}: means in the same row with different superscript are significant (P<0.05), T1=0%VATD meal; T2=5%VATD meal; T3=10%VATD meal; T4=15VATD meal; T5=20%VATD meal.

Table 5. Nitrogen utilization of WAD goats fed *Tithonia diversifolia* and *Vernonia amygdalina* leaf meal based concentrates.

Parameters	Diets					P-value	SEM
	T1	T2	T3	T4	T5		
Nitrogen intake g/day	14.32^{b}	14.16^{b}	14.76^{ab}	15.10^{a}	14.88^{ab}	0.0374	0.21
Nitrogen excretion (g/day)							
Faecal	1.82	1.77	1.89	1.95	1.85	0.9307	0.02
Urine	0.07	0.06	0.07	0.07	0.07	0.9893	0.00
Nitrogen balance	12.43	12.33	12.80	12.30	12.96	0.0607	0.23
Nitrogen retention (%)	86.80^{a}	87.08^{a}	86.72^{a}	81.46^{b}	87.10^{a}	0.0020	0.22

^{abc} : means in the same column with different superscript are significant (p<0.05). Ti= 0% VTL meal, T2= 5% VTL meal, T3= 10%VTL meal, T4= 15% VTL meal, T5= 20% VTL meal.

N-balance and N-retention which are functions of nitrogen ingested and digested (Okoruwa et al., 2013) were positive in this study. Nitrogen balance values were similar in all diets. The positive nitrogen balance observed in all treatment groups suggest that the nitrogen absorbed, which is the difference between nitrogen intake and faecal nitrogen was well tolerated and utilized by the animals. Nitrogen retention (%) was lower (P<0.05) in goats fed T4 than in others, probably due to higher nitrogen intake and higher faecal N excretion. This observation corroborates the reports of Alli-Balogun et al. (2003) when they fed cassava foliage to sheep and observed high faecal and urinary nitrogen, which also led to poor nitrogen retention among the experimental animals.

Conclusion

1) *T. diversifolia* and *V. amygdalina* leafmeal is high enough in crude protein and comparable to those found in Wheat offal and PKC.
2) VATD leafmeal based diets can meet the maintenance nitrogen requirements of WAD goats, as all the experimental animals were observed to be in positive nitrogen balance.
3) WAD goats fed *T. diversifolia* and *V. amygdalina* leafmeal based concentrates as supplement to low protein *P. maximum* hay performed better on diets with concentrates containing leaf meals at levels not exceeding 10%.

Conflict of Interests

The authors have not declared any conflict of interest.

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Full Length Research Paper

Germination and seed traits variations among West African provenances of *Moringa oleifera* Lam. (Burkina Faso)

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Moringa oleifera **is a fruit species of economic interest to West African smallholder growers. To generate benefits to poor rural communities, improved knowledge is needed on plantation management and selection of the most reliable seed sources. The aim of the study was to investigate variation in seed traits and germination rates among 12 provenances of *M. oleifera* from West Africa in the women gardening center named "Amicale des Forestières du Burkina Faso (AMIFOB)" located at Ouagadougou, Burkina Faso (12°7'32''N, 01°40'24''W). The authors conducted an analysis of variance, a principal component analysis on seed traits variables (length, thickness and weight) and germination rates at 5 and 12 days after seed sowing, and finally ascending hierarchical classification based on similarity indices. The results showed significant variations (*P<0.05)* among provenances in seed traits: Ouahigouya provenance in the Sahelian area of Burkina Faso had the largest and heaviest seeds. Germination rate was significantly different after 5 days for Ouahigouya, Ouagadougou, Koudougou, Dano and Tamale provenances (*P*<0.05). Five days after seed sowing, the Sahelian provenance (Ouahigouya) recorded the greatest and fastest germination rate of 63%. Correlation analyses revealed no significant links between germination rate in 5 and 12 days after sowing and seed sizes. Seed traits and germination rates did not show clear cut distinct groups between Sahelian, Sudanian and sub Equatorial provenances. This research output provides an evidence of the genetic variability among *M. oleifera* provenances and hence the potential for future tree improvement programme.**

Key words: Seed variability, agroclimatic zone, Burkina Faso.

INTRODUCTION

Moringa oleifera Lam. or 'drumstick tree' is a member of the *Moringaceae* family, which grows throughout the tropics and is native to the sub-Himalayan tracts of north-western India, Pakistan, Bangladesh and Afghanistan

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(Makkar and Becker, 1997). The tree has a height of 7 to 12 m up to the crown. The leaves are twice or thrice pinnate and spirally arranged. The flowers are bisexual, white or cream colored with yellow stamens, and the fruit is a three sided or nearly cylindrical capsule. The seed is round, dark chestnut-colored, and with 3 white wings that facilitate spreading by the wind in natural conditions. *Moringa* can be propagated either by using seeds or cuttings (Morton, 1991). Fruits, seeds, leaves and flowers are consumed by humans as nutritious vegetables. *M. oleifera* leaves are known to be an excellent source of vitamins, minerals and protein and are used to fight against malnutrition and its associated diseases including blindness (Asante et al., 2014). In West Africa, leaves are commonly used to make sauces. It is grown in all types of soil, from acid to alkaline (Galdima et al., 2013) and can tolerate up to 6 months of dry season reasonably well. The tree grows at different altitudes from 0 to 1800 m.a.s.l. and rainfall between 500 and 1500 mm per year.

M. oleifera is planted and smallholder growers grow subsistence and cash crops in their rainfed, complex and resource deprived fields often combining the cultivation of the crops with scattered multipurpose trees and realizing a wide range of benefits (Mudyiwa et al., 2013). It is one of such multipurpose tree of global interest and is grown in combination with agricultural and horticultural crops by smallholder growers and this give growers a wide range of benefits (Mudyiwa et al., 2013). It is a suitable tree for traditional agroforestry in the home because of its versatility (Nduwayezu et al., 2007).

In Burkina Faso, the seeds sources for planting are coming from various agro ecological zones. For example, more than 80% of women growers used non certified seeds as plantation materials to produce *M. oleifera* leaves because they do not access the high cost of certified one. The demand of plantation material is most likely growing in the future due to increasing demand of the potential market. Better plantation and nursery management would generate benefits to poor rural communities, since villages and hamlets hold their own local nursery. However, before plantations can be recommended on a large-scale, improved knowledge is needed on plantation management and selection of the most reliable seed sources. There are noticeable differences among *M. oleifera* agroecological ecotypes concerning growth performances (Edward et al., 2014) which have considerable impact on the economic return. Differences among geographic sources in tree species are often substantial and economic improvement can be made by an appropriate provenance selection (Wright et al., 1976).

Altitudinal variations in seed and seedling characters of tree species have been reported by various studies (Gera et al., 2000). However, for the utilization of observed variation in species, it is a prerequisite to know the extent of variation and also whether it is due to genetic or environmental factors. Hence, information on variation in the desirable parameters and their correlation is vital for any breeding programme (Fenner and Thompson, 2005).

Little is known about the role of *M. oleifera* seed size. Seed size has been regarded as an important plant property (Fenner, 1991). Considering the presence in various ranges of climate and soil conditions, and different agro ecological zones with different ecotypes may show contrasting performances (Aronson et al., 1993).

In the current study, the authors investigated the seed size among 12 provenances of *M. oleifera* from four agroecological zones of West Africa. They hypothesized that the seed size and germination rates correlated with geoclimatic data of seed collection sites.

MATERIALS AND METHODS

Study site

The study was conducted at the women gardening center named “Amicale des Forestières du Burkina Faso (AMIFOB)” located at Ouagadougou, Burkina Faso (12°7’32’’N, 01°40’24’’W). The rainfall is uni-modal with a mean annual rainfall of the last 15 years data from the nearest meteorological station in Ouagadougou, of 800 mm.year^{-1}, and the mean temperature of 28.5°C month^{-1} (Figure 1). Soils are sandy clay to clay-sandy Ferruginous leached with very low nutrient content according to French soil classification (Pallo et al., 2009). The common natural vegetation found at Ouagadougou is described as semi-deciduous open woodland. Main genera include, *Eucalyptus, Azadirachta, Mangifera, Vitellaria, Lannea, Piliostigma, Acacia., Ziziphus, Tamarindus* and *Combretum*.

Seed sources, experimental design and establishment

The experiment included twelve *M. oleifera* provenances: 1 provenance from Segou (Mali), 1 from Niangon-Lokoua, Abidjan (Ivory Coast), 1 from Tamale (Ghana) and 9 from Burkina Faso (Ouahigouya, Dano, Gaoua, Ouagadougou, Fada N’Gourma, Dédougou, Bobo-Dioulasso, Koudougou, Centre National de Semences Forestières-CNSF) (Figure 2).

In this paper, provenances were referred to four climate areas according to their agro-ecological characteristics (Sahelian, sub Equatorial, Sudanian and north Sudanian) (Table 1). Seeds were collected in 2014 in plantation farmland from at least 12 mother trees per provenance.

Trial was planted in a randomized complete block design (RCBD) with three replications. Each plot represented a provenance planted at 5 x 6 rows in a contiguous arrangement of 20 x 20 cm (Figure 3). Plot measured 10 x 1 m and contained 30 trees and the distances between blocks were 2 m. Seed samples were pretreated with water for 24 h and sown in June 1st 2014 in a prepared and cleaned soil using hand hoes. Weeding was done twice during the rainy season. Watering was done once a day in the morning. The seedlings were grown without fertilizers and chemical control.

Data collection and analysis

To investigate the variability in seed parameters (length, thickness and weight), each provenance was represented by 240 randomly selected seeds, assessed in four replications of 60 seeds each. Each seed weight was determined by weighing three random samples of 60 seeds each. By the end of 2 weeks, the experiment

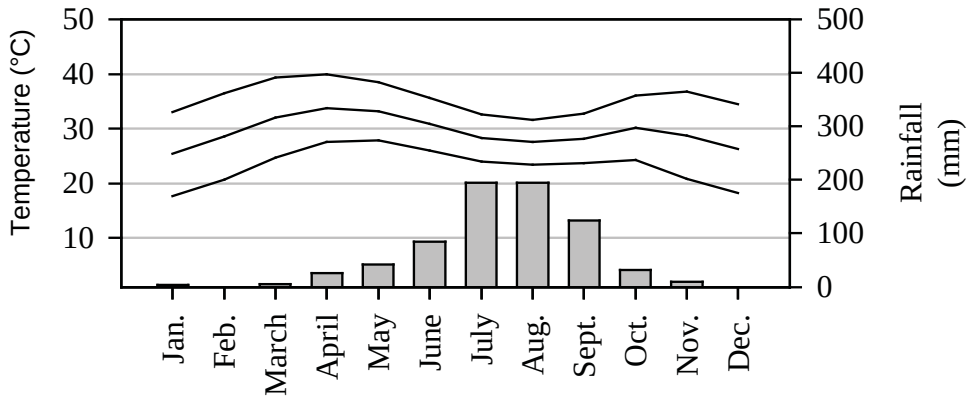

Figure 1. Monthly temperature (min., mean, max.) and precipitation level measured between 2000 and 2015 at Ouagadougou. Data were obtained from http://www.infoclimat.fr/stations-meteo/climato-moyennes-records.

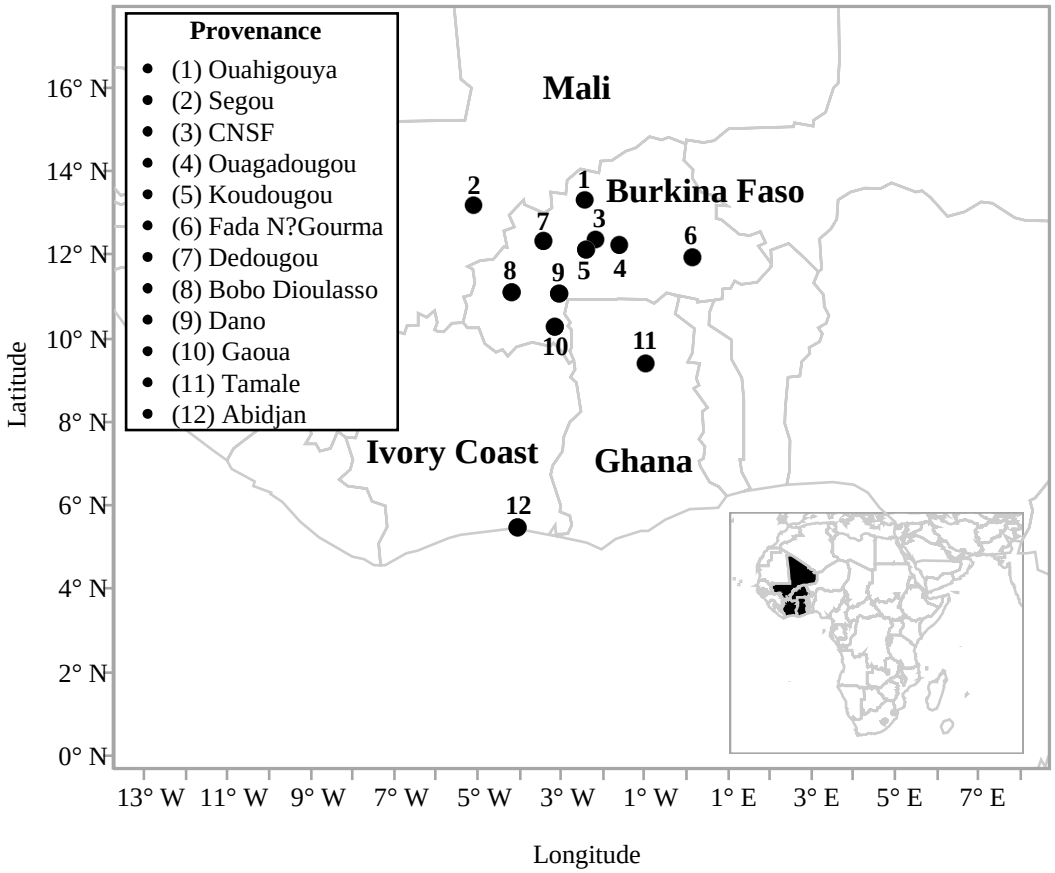

Figure 2. Provenances localizations.

had been measured twice at effective ages of 5 and 12 days since seed sowing, providing data for the assessment of germination rates of the twelve provenances. All statistical analysis were carried out using ANOVA performed with the MIXED procedure of JMP® Pro 11.1.1 (SAS Institute, Cary, NC, USA). Normality and homoscedasticity were graphically verified on residual plots of the linear models (Quinn and Keough, 2002). When effects were significant, the Tukey-Kramer test was used for multiple mean comparisons. Statistical significance was fixed at 0.05, and only significant results are presented in graphs. A factorial analysis was conducted to determine the relationship of seeds, germination and their provenances of origin.

Table 1. Characteristics of the provinces, ecological zones and seed sources of *M. oleifera.*

Prov. code	Ecological Zone	Provenance	Country	Latitude	Longitude	Altitude (m)	Average rainfall (mm/year)	Mother trees number
1	Sahelian	Ouahigouya	Burkina Faso	13°30'4"N	2°24'31"W	306	500	24
2	North Sudanian	Segou	Mali	13°22'05"N	5°16'24"	294	500	35
3	North Sudanian	CNSF	Burkina Faso	12°30'07 N	2°07'34"W	304	800	35
4	North Sudanian	Ouagadougou	Burkina Faso	12°21'58"N	1°31'05"W	315	800	15
5	North Sudanian	Koudougou	Burkina Faso	12°15'04"N	2°22'28"W	308	800	12
6	North Sudanian	Fada N'Gourma	Burkina Faso	12°03'41"N	0°21'30"E	300	900	26
7	Sudanian	Dedougou	Burkina Faso	12°28'00"N	3°28'00"W	300	1100	16
8	Sudanian	Bobo Dioulasso	Burkina Faso	11°11′00″N	4°17′00″W	339	950	20
9	Sudanian	Dano	Burkina Faso	11°9'0"N	3°4'0"W	287	950	30
10	Sudanian	Gaoua	Burkina Faso	10°19'12"N	3°10'12"W	319	1000	17
11	Sudanian	Tamalé	Ghana	9°24'27"N	00°51'12"W	169	1100	15
12	Sub-Equatorial	Abidjan	Ivory Coast	5°18'28"N	4°6'19"W	73	2000	18

Figure 3. An experimental trial of *M. oleifera* provenance. Photo DAO MCE, Ouagadougou, July 2014.

RESULTS

Seed size traits

Analysis of variance showed highly significant differences among *M. oleifera* provenances within ecological zones for seed length, thickness and seed weight ($P < 0.0001$) (Figure 4). Estimates of variance components indicated that the inter provenances effects varied from 42 to 45% of the total variation for all seed characters (Table 2). The

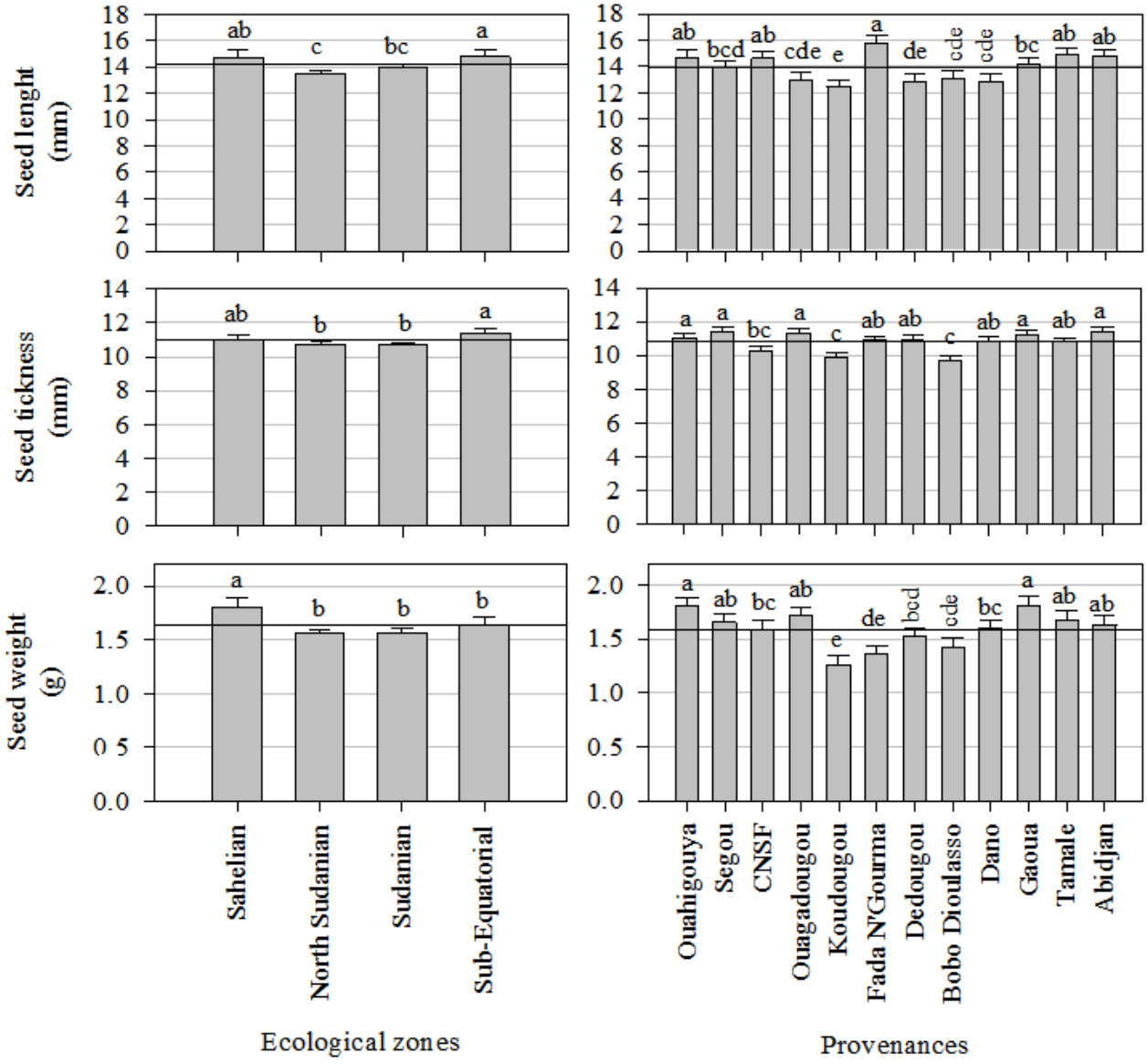

Figure 4. Seed characteristics of the12 provenances of *M. oleifera,* least-square-means + 95% confidence intervals. Different letters indicate significant differences ($p<0.05$, Tukey HSD test).

Table 2. Estimates of variance components, expressed as percentage of the total variation, and provenance heritability (h^2_p) within ecological zone for seed size traits of the 12 *M. oleifera* provenances.

Variables	Ecol. Zone	Prov[Ecol. Zone]	Residuals	h^2_p
Seed length	0	42.97***	57.03***	0.94
Seed thickness	0	43.75***	56.25***	0.94
Seed weight	0	45.20***	54.80***	0.94

*** $p < 0.001$.

provenance heritability for all seed characters was relatively high (0.94).

Maximum mean values for seed length (15.8 mm) were found in Fada N'Gourma, for seed thickness (11 mm) in 5 provenances (Ouahigouya, Ségou, Koudougou, Gaoua and Abidjan) and for seed weight (1.8 g) in Ouahigouya

Table 3. Eigenvectors (W) and loadings (L) of the PC axis from PC analysis of the 12 *M. oleifera* provenances. Eigenvalues and their contribution to total variation are listed at the bottom of columns.

Variable	PCA 1		PCA 2		PCA 3	
	W	L	W	L	W	L
Latitude	0.506	0.900	0.257	0.330	0.007	0.008
Longitude	-0.019	-0.033	0.100	0.128	0.780	0.870
Altitude	0.534	0.949	0.118	0.152	-0.004	-0.005
Average rainfall	-0.476	-0.845	-0.338	-0.434	-0.016	-0.017
Germination rate	0.213	0.379	0.026	0.034	0.248	0.277
Seed length	-0.261	-0.464	0.295	0.380	0.478	0.533
Seed thickness	-0.291	-0.517	0.566	0.728	-0.067	-0.075
Seed weight	-0.186	-0.330	0.622	0.800	-0.311	-0.347
Eigen value	3.16		1.65		1.24	
Percent of total	39		21		16	

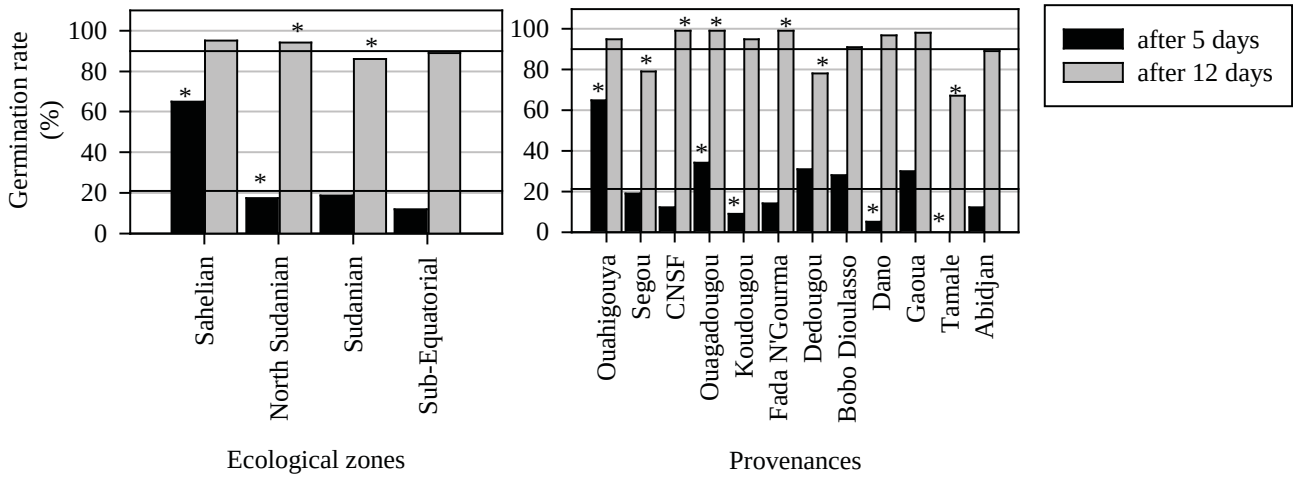

Figure 5. Cumulative germination rate (%) after 5 and 12 days of the 12 provenances of *M. oleifera*. * indicate significant differences ($p < 0.05$, ANOM method) with the means at the two evaluation days (5 and 12 days).

and Gaoua provenances (Table 3). Ranking of provenances based on mean seed length, thickness and weight indicated consistently lower values for Koudougou and Bobo Dioulasso (Table 3). Ouahigouya (Sahelian) and Gaoua (south Sudanian) were characterized by heaviest and largest seeds, while Koudougou (north Sudanian) and Bobo Dioulasso (south Sudanian) provenances were the shortest, tiniest and lightest seeds. The provenances were classified in descending order of magnitude by high, intermediate and low groups of seed characters as follows:

Seed length: Fada N'Gourma > Ouahigouya, CNSF, Abidjan, Gaoua, Ségou and Tamale > Ouagadougou, Dano, Koudougou and Bobo Dioulasso
Seed thickness: Ouahigouya, Gaoua, Ségou, Ouagadougou, Tamale > Fada N'Gourma, Dano, CNSF and Tamale > Koudougou and Bobo Dioulasso
Seed weight: Ouahigouya, Gaoua > Ségou, Ouagadougou, Tamale, Abidjan, CNSF, Dano > Fada N'Gourma, Koudougou and Bobo Dioulasso.

Seed germination

Seeds started germinating 5 days after sowing and after day 12 no seeds germinated. The overall means of germination rate of *M. oleifera* seeds under experimental conditions were 21 and 90% after 5 and 12 days after sowing, respectively (Figure 5). The cumulative germination rate was significantly different after 5 days for Ouahigouya, Ouagadougou, Koudougou, Dano and Tamale ($P < 0.05$) (Figure 5). Five days after seed sowing, Ouahigouya provenance recorded the greatest

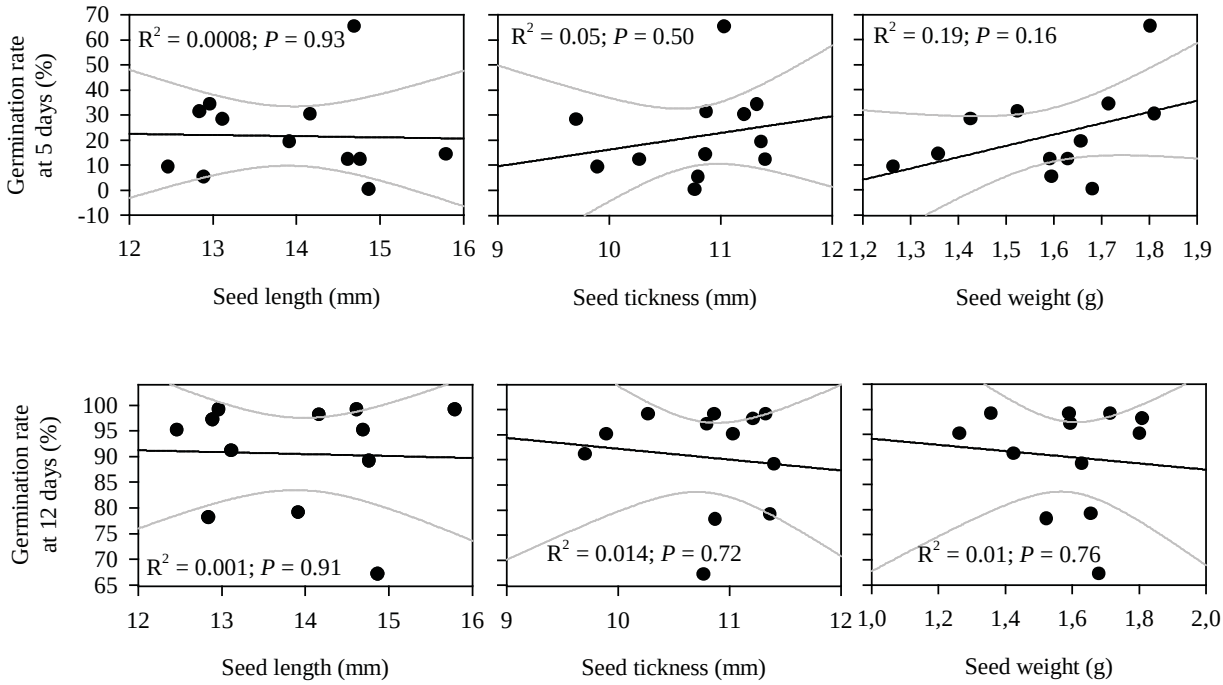

Figure 6. Linear regression between germination rate at 5 and 12 days and seed length, seed thickness and seed weight of the *M. oleifera*. R2 is the regression correlation and P the prob. value of the ANOVA for the simple linear regression of Y on X.

and fastest germination rate of 63%.

The response of seeds showed great variability depending on provenance and ecological zone (Figure 5). Significant effects were found between ecological zones ($P < 0.05$). After 5 days, germination rate of the provenance in the Sahelian zone (Ouahigouya) was 3 times higher (more than 60%). Within the ecological zone, no significant effects were observed at both dates 5 and 12 days after sowing with Abidjan (sub Equatorial), Gaoua and Bobo Dioulasso (south sudanian) provenances (Figure 3). The highest final germination percentages of 100% were observed with provenances from the north Sudanian zone of Burkina Faso (CNSF, Ouagadougou and Fada N'Gourma). The final lowest germination rates were observed in Tamale (68%), Dedougou (78%) and Segou (80%) provenances (Figure 5).

Correlations of seed characteristics with germination rates

Correlation analyses revealed no significant links between germination rate in 5 and 12 days after sowing and seed length ($r^2 = 0.00$; $P = 0.93$ and $r^2 = 0.00$; $P = 0.91$ respectively in 5 and 12 days after seed sowing), seed thickness ($r^2 = 0.05$; $P = 0.50$ and $r^2 = 0.01$; $P = 0.72$, respectively in 5 and 12 days after seed sowing) and seed weight ($r^2 = 0.19$; $P = 0.16$ and $r^2 = 0.01$; $P = 0.76$, respectively in 5 and 12 days after seed sowing) (Figure 6).

Multivariate analysis of seed characters, germination and geoclimatic data of provenance origin

The principal component analysis (PCA) performed on the characteristics of seeds and twelve-day-old seedlings showed that the first three principal axes explained 77% of the observed variability (Axis 1: 40%, axis 2: 21% axis 3: 16%) (Table 3). The axis 1 variables were influenced by the average rain and latitude and altitude of the provenances origin and constituted the axis of geoclimatic characters of the provenance origin. The axis 2 explained the contribution to total variation of variables seed thickness and weight. This axis may be defined as the center of the seed characteristics. The axis 3 was mainly explained by the seed length, germination rate and longitude and was defined as the axis of the seed length (Figure 7).

By overlaying the correlation circles of variables and map of overall seed provenances with the main axes, it appears that provenances 11 (Tamale), and 12 (Abidjan) are highly correlated with average rainfall of the axis 1 and located at the negative side of the axis 1 (Figure 7). Axe 3, the provenance 12 and 11 and the variables seed thickness and weight were located on the positive side of the axis 3 while the provenance 6 and the variable seed length were on the opposite side. A hierarchical cluster

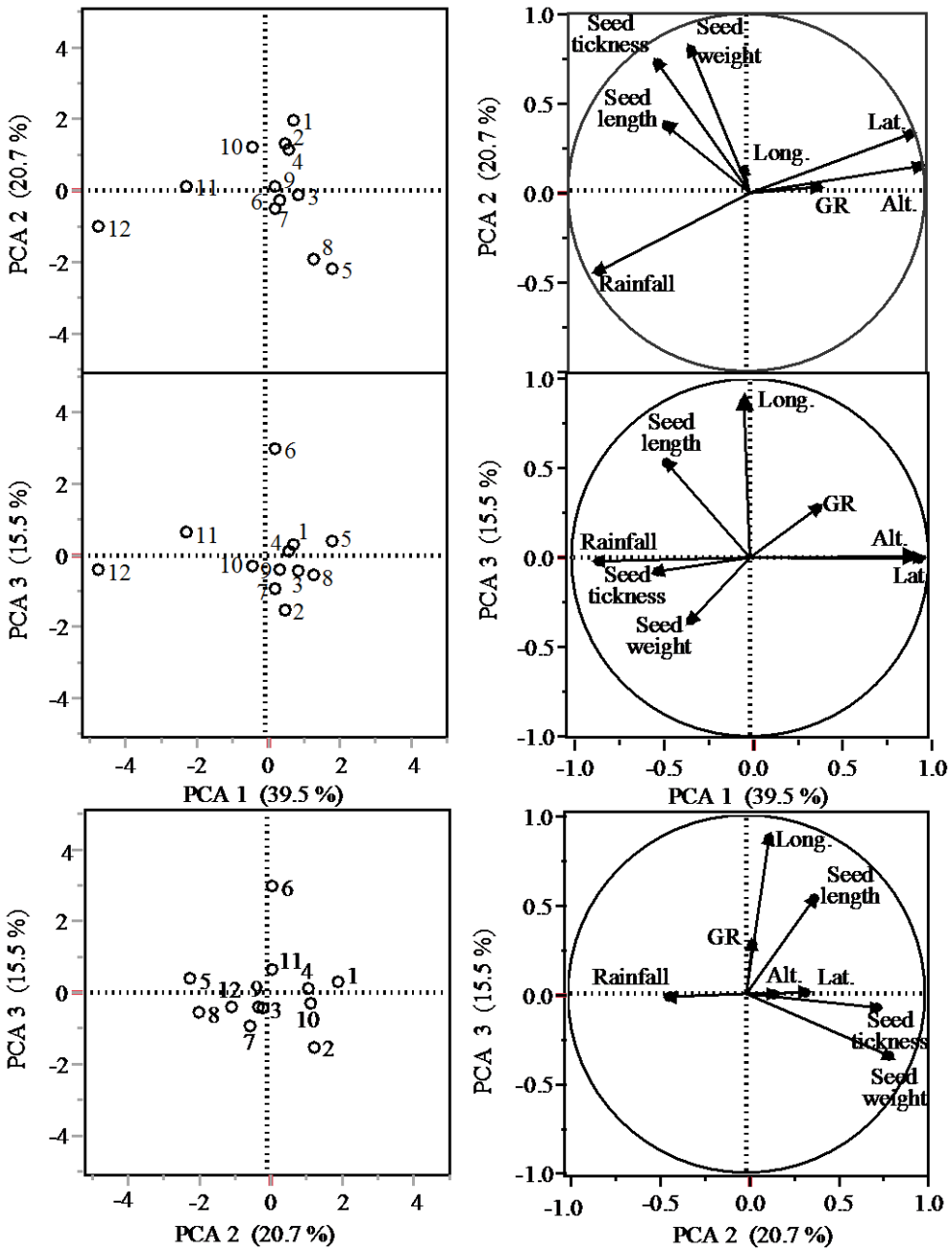

Figure 7. Principal component analysis of the 12 *M. oleifera* provenances based on a correlation matrix of association between seed characteristics and environmental variables. GR: final germination rate. [1] Ouahigouya, [2] Segou, [3] CNSF, [4] Ouagadougou, [5] Koudougou, [6] Fada, [7] Dedougou, [8] Bobo, [9] Dano, [10] Gaoua, [11] Tamale and [12] Abidjan.

analysis dendrogram showned no clear identified provenance groups (Figure 8).

DISCUSSION

This study was conducted in Ouagadougou on 12 provenances of *M. oleifera* from West African, and tested the hypothesis that seed size and germination rates were correlated with geoclimatic data of seed collection sites. The results revealed clear differences among the 12 *M. oleifera* provenances in terms of seed weight, length and thickness. Seeds collected from Ouahigouya in the sahel zone were heavy and large with faster germination rate in the natural environmental conditions. In contrast, seeds collected in provenances located in more humid zones

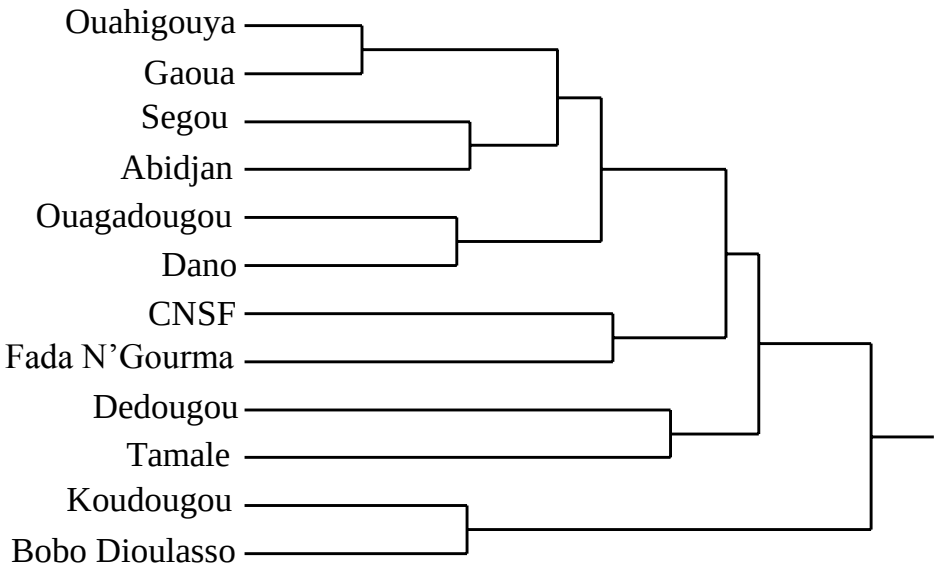

Figure 8. Hierarchical cluster dendrogram of the 12 *M. oleifera* provenances based on seed characteristic and final germination rate.

(north and south sudanian, sub equatorial) were small, less heavy and thin.

Seed size, is regarded as key factor of reproductive strategy because of its important role in establishment of the juvenile phase of tree life cycle. For example, large seed size has been documented to prolong dormancy during unfavorable light conditions, development of large amounts of photosynthetic tissue, allowing quick seedling growth and dispersal modes (Mamo et al., 2006). According to Roach et al. (1987), in very wet areas where rainfalls vary between 1000 and 1500 mm · year^{-1}, the seeds would be generally large. In this study, our results in contrary showed that the provenances seeds from wetter areas were smaller. The observed seed sizes differences are rather due to the effects of the genetics and would therefore not be environmentally based (Roach et al., 1987). Much of the total variation in seed traits are due to genetic influence as evidence from high heritability estimates (>0.90%). These genetic variations in seed traits have been reported for several tree species (Bonito et al., 2011; Khan et al., 2012; Ky-Dembele et al, 2014). Our findings in *M. oleifera* are in conformity with these research findings. Similar genetic variation in seed sides has been reported for several tree species (Diallo et al., 2010; Ky-Dembele et al., 2014).

The present study revealed the existence of significant differences of germination rates between provenances 5 days after seeds sowing. Ouahigouya provenance from the dry agroclimatic area recorded the greatest and fastest germination rate of 63%. This variation among provenances at early stage of germination indicated that greater and heavy seeds of Ouahigouya provenance results in faster seedlings growth. Rapid germination and subsequent seedling growth are, therefore, key phenotypes of vigorous seeds that are known to differ with genetic background (Bettey et al., 2000). Thus, a vigorous seed must possess key trait of rapid germination to establish seedlings across a wide range of environments (Khan et al., 2012).

In the present study, the superior performance of Ouahigouya in seed size and germination rate suggests that conservation and management of this provenance as seed source could improve *M. oleifera* in the north Sudanian and Sahelian areas of Burkina Faso. In most plant species, seeds vary in their degree of germinability between and within populations and between and within individuals (Nyoka et al., 2015). Some of this variation can be of genetic origin, but much of it is known to be environmental, that is caused by the local conditions under which the seed matured (Mamo et al., 2006).

The study revealed no significant correlations between seed characters and germination capacity. The finding suggests that seed weight, length and thickness have little importance in predicting the germinability of *Moringa* seeds. This was already demonstrated by Fenner and Thompson (2005) and Kazmi et al. (2012). Thus, seed sides are beneficial for the establishment of seedlings, but there appear to be no consistent correlation between seed and germination characteristics.

The hierarchical cluster analysis dendrogram revealed no clear difference between provenances. Thus, no groups of provenances were markedly discernable for seed characters, germination and geoclimatic data of provenance origin. This method of grouping of seed provenances indicated that distribution into different groups did not follow any pattern with regard to

agroclimatic seed sources.

Conclusion

This study is the first in West African to characterize *Moringa* seed sizes and germination rates. The results indicate that variability exist among provenances in seed sizes of *M. oleifera* from different agroclimatic zones of West Africa. Based on observed seed sizes and germination rates, the most prominent provenance is Ouahigouya. Seeds collected from this provenance are heavy and large with faster germination rate; thus this provenance might be considered for an eventual *M. oleifera* improvement program to enhance productivity in plantations in north Sudanian and Sahelian areas of Burkina Faso.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Quantification and conservation status of forest fragments in part of Brazilian Atlantic Forest

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Forest fragments present shapes and sizes that become them more or less susceptible to external factors, which can be measured by means of ecological indexes. The aim of this research was to diagnose conservation status and to quantify forest fragments in part of the Brazilian Atlantic Forest. Forest fragments were mapped and landscape ecology metrics were calculated, including area size, fractal dimension index, and edge index. These three metrics were modeled using the Weibull-3P probabilistic function in order to quantify fragments in a specified range. A bivariate function also was applied relating the indexes with fragment area size. The diagnosis revealed that the study area is highly fragmented, since the forested area covers only 13.7% of the total area. About 89% of the forest fragments have area from 10 to 100 ha, whereas fragments larger than 100 ha totalize about 11%. The metrics allowed discriminating the forest fragments by their conservation status. This study suggests that smaller fragments should be managed as stepping stones to the larger ones. As conclusion, larger forest fragments present more complex shapes and high edge effect. Thus, smaller forest fragments present variability of shapes from simple up to the more complex ones, besides edge effect much more variable than the larger fragments. The frequency of fragments in relation to the studied variables follows a normal. This means that well-conserved forest fragments, that is, with biggest areas, lower edge effects, and more rounded shape, are the scantiest ones.

Key words: Forest area size, edge index, fractal dimension index, probability density function.

INTRODUCTION

The human pressure on forests in Brazil has occurred since the beginning of its colonization. The Atlantic Forest biome was the first to suffer strong effects on the vegetation, which has increasingly been fragmented.

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Forest fragmentation can be defined as any area of natural vegetation interrupted by human barriers such as roads and crops, or natural barriers such as rivers and lakes, which can reduce the flow of animals, pollens and seeds (Forman and Godron, 1981).

The consequences caused by fragmentation are not yet fully understood, but one of them certainly is the decreasing of areas of the original habitats, which affects genic flow and abundance of biodiversity (Lawrence and Vandecar, 2015; Cumming et al., 2012). Besides the area size, the dynamics of forest fragments correlate with their shape, degree of insulation, type of neighborhood and historic of disturbance, interfering in the composition, as well as in vegetation and wildlife survival (Souza et al., 2008; Hill and Curran, 2003; Viana and Pinheiro, 1998).

In landscape ecology concept, the forest fragmentation consists of a mosaic of patches contained in a matrix, in which its structure suffers external influences such as wind, fire, and human intervention. In this sense, ecological metrics are used to relate perimeter and area, reflecting the level of complexity and edge of patches. Analogously, considering the assumption that the circle is the geometric shape with a smaller perimeter for a certain area, the most rounded forest fragment has the lower edge effect, besides its shape is less complex (Broadbent et al., 2008; Couto, 2004; Forman and Godron, 1981).

Some ecological metrics as area size and perimeter-area indexes were adopted by Santos et al. (2016) aiming to select forest fragments to collect forest seeds. The fragments were staggered based on their potential genetic richness, since indexes of edge and shape provide estimates of the degree of susceptibility to external disturbances.

The seed selection and collection without adopting appropriate technical criteria result in reduction of physiological and genetic quality, including germination and development of biological immunity of forest seeds and seedlings (Piña-Rodrigues et al., 2007).

Few studies are developed aiming to contribute to the management of natural forests, mainly with respect to forest fragments with sufficient condition to ensure genetic quality of forest seeds. From the mapping of forest fragments in part of the Brazilian Atlantic Forest, this study was conducted with the hypothesis that the pressure from human activities reduced substantially the well-conserved forests in the region.

The aim of this study was: (1) to present a diagnosis of conservation status of forest fragments using as criteria landscape ecology metrics and (2) to generate probabilistic models to quantify forest fragments.

MATERIALS AND METHODS

Study area and forest mapping

The study area encompasses part of two states of southeastern Brazil. The region is located in the Itapemirim river basin and surrounding Caparaó National Park, which corresponded to a buffer covering an area of 10 km from the Park's boundaries. The basin and the park are included in the southeastern part of the state of Minas Gerais, and in the southern part of the state of Espirito Santo.

The geographical localization is between parallels 20° 48' and 21° 05' south and meridians 40° 48' and 41° 58' west. The region encompasses 30 counties and occupies a total area of around 6,640 km².

According to Köppen classification, the study area has three climates: Aw or savanna climate, Cwa, and Cwb. The vegetation belongs to Atlantic Forest biome with predominant formation of dense rainforest, semi-deciduous forest, sandbanks, and mangrove.

The mapping of forest fragments was performed as a stage of a project called "Forest Seeds Network of Surrounding the Caparaó and of Itapemirim River Basin", in which fragments with area larger than 10 hectares (ha) were delimited, since smaller fragments are more susceptible to biodiversity changes and inbreeding (Santos et al., 2016).

Coffee production and cattle grazing are the most important agricultural activities in the region; therefore, coffee plantations and grasslands are the main causes of pressure on the vegetation (Mannigel, 2008; Sales et al., 2013). Pirovani (2010) describes that coffee cultivations intensified in the second half of the 19th century, but after that some factors (as loss of fertility) contributed to coffee areas be changed to grasslands.

1:15,000 scale and 1-m spatial resolution digital aerial photographs obtained in 2007 from the State Institute for the Environment were used. The ArcGIS software (version 9.3) was used to assist the digitization of fragments adopting 1:2,500 scale and after to estimate areas and perimeters.

Landscape ecology metrics

After complete the mapping, landscape ecology metrics were calculated to each forest fragment, whose values were used as criterion to identify those well-conserved forest fragments (Figure 1).

The metrics corresponded to fractal dimension index (Equation 1): it refers to the shape complexity of forest fragments and assumes lower values for simple and regular shapes, whereas the higher values correspond to more complex and convoluted shapes (Santos et al., 2016; Couto, 2004).

$$FDI = \frac{2\ln\left(\frac{P}{4}\right)}{\ln(A)} \quad (1)$$

The second metric was the circularity or edge index (Equation 2): it reflects the edge effect and susceptibility of forest fragments to external factors (Santos et al., 2016; Viana and Pinheiro, 1998). Circular form assumes one as maximum value. This index is expressed by:

$$EI = \sqrt{\frac{A}{Ac}} \quad (2)$$

where FDI is the fractal dimension index; EI is the edge index; P is the perimeter of the fragment, in m; A is the area size, in m²; Ac is the area (m²) of a circle with same perimeter; ln is the natural logarithm.

Finally, the third landscape ecology metric was area size (ha) of the fragment. The metrics were modeled with the Weibull-3P

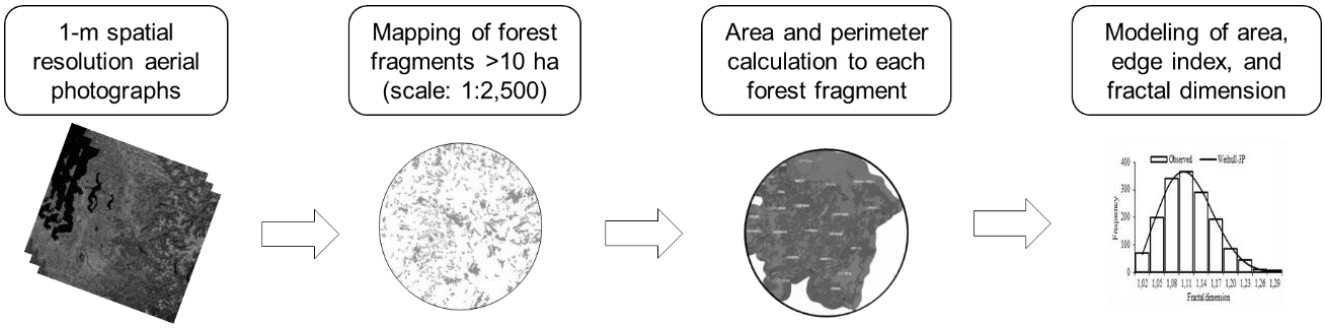

Figure 1. Methodological procedure to model landscape ecology metrics of forest fragments.

Table 1. Descriptive statistics of the variable area size (ha) of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

Group	Minimum	Maximum	Average	Median	CV (%)	No. of fragments
10-100 ha	10.0	99.8	29.0	21.4	67.1	1,438
100-500 ha	100.2	498.2	195.7	155.1	50.8	155
> 500 ha	537.7	4,293.1	1,098.3	800.6	72.1	27
Total	10.0	4,293.1	62.8	24.2	286.2	1,620

CV: Coefficient of variation.

probability density function (pdf) to quantify the occurrence of forest fragments in a given interval. The procedure was repeated to the three metrics. The Weibull-3P pdf (Equation 3) is expressed by:

$$f(x) = \frac{c}{b}\left(\frac{x-a}{b}\right)^{c-1} e^{-\left(\frac{x-a}{b}\right)^{c}} \quad (3)$$

where x is the variable of interest; a, b and c are the location, shape and scale parameters of the model respectively; *e* is the exponential.

In relation to the variable area size, the pdf was adjusted by groups; being from 10 to 100 ha, 100 to 500 ha and larger than 500 ha. This separation was required due to the high variation of area sizes when encompasses all of them together. Table 1 presents the main descriptive statistics in relation to the groups of area size.

To the other variables (DFI and EI), these three groups were disregarded due to their low variations. The modeling counted on ten frequency classes, except the group larger than 500 ha, whose number of classes was reduced to six, due to its low number of observations.

Thus, these functions could be used to estimate only one variable. Then, bivariate normal function also was applied to the bivariate case (Pennacchi et al., 2006). The generalization of the univariate normal function (Equation 4) leads to the multivariate normal function (Equation 5):

$$f(x) = \frac{1}{(2\pi)^{1/2}\sigma} e^{-\frac{1}{2}\left[\left(\frac{x-\mu}{\sigma}\right)^2\right]} \quad (4)$$

with $x \in R$, $\mu \in R$ and $\sigma \in R^{+}$.

The generalization allows the combination of p normal variables in the function:

$$f(x_1, x_2, \ldots, x_p) = \frac{1}{(2\pi)^{p/2}\sigma_1\sigma_2\ldots\sigma_p} e^{-\frac{1}{2}\left[\left(\frac{x_1-\mu_1}{\sigma_1}\right)^2 + \left(\frac{x_2-\mu_2}{\sigma_2}\right)^2 + \ldots + \left(\frac{x_p-\mu_p}{\sigma_p}\right)^2\right]} \quad (5)$$

where x_i is the variables of the model; σ_i and μ_i is the parameters of the model; p is the number of variables; *e* is the base of natural logarithm; π is the "pi" constant.

Integrating $f(x_1, x_2)$, it was possible to obtain the probability of occurrence or percentage of forest fragments in a desired interval of area size and of fractal dimension or of edge index. Through the bivariate models, we drew 3D graphs relating area size and one of the indexes, by using the software Matlab version R2012a.

The normal bivariate function presupposes that linear combinations of the components $\underline{X}$ follow a normal distribution N p-variate with mean vector $\underline{\mu}$ and covariance matrix Σ $\left[\underline{X} \sim N_p(\underline{\mu}, \Sigma)\right]$ and then, the Chi-square (χ^2) test (Equation 6) for normality was performed at 50% of probability.

$$P\left[\underline{X}:(\underline{X}-\overline{\underline{X}})'S^{-1}(\underline{X}-\overline{\underline{X}}) \leq \chi_p^2(0.5)\right] \quad (6)$$

where $\overline{\underline{X}}$ estimates $\underline{\mu}$ and S estimates Σ.

The initial hypothesis (H_0) corresponds to existence of data normality while the alternative hypothesis (H_1) corresponds to non-normality.

Accuracy and goodness of fit

The accuracy of the Weibull-3P probability density function (pdf)

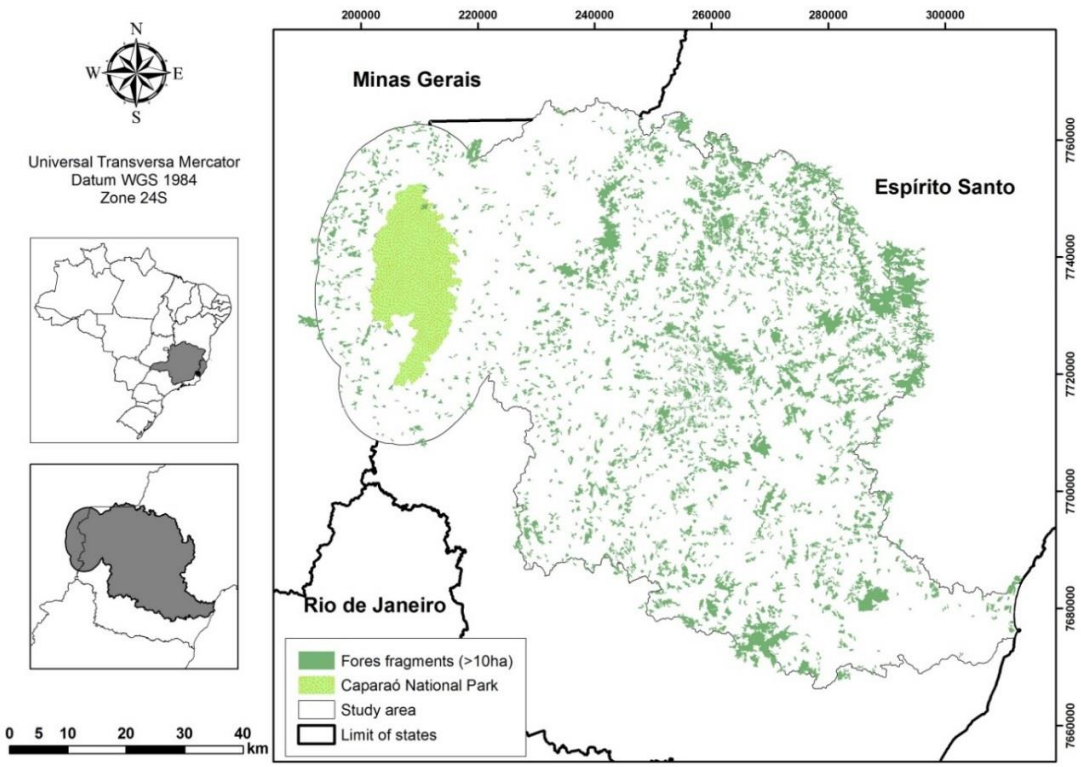

Figure 2. Forest fragments with area larger than 10 ha in the Itapemirim river basin and surrounding the Caparaó National Park.

was evaluated by adopting the standard error of estimate (SEE) in percentage (Equation 7). The goodness of fit was verified by the Kolmogorov-Smirnov (KS) test (Equation 8) at 95% probability level (Razali and Wah, 2011).

$$SEE\ (\%) = \sqrt{\frac{\sum_{i=1}^{n}(Y_i - \hat{y}_i)^2}{n - p}} \cdot \frac{100}{\bar{y}} \quad (7)$$

$$KS = \sup_x |Fo(x) - Fe(x)| \quad (8)$$

where Fo is the observed cumulative frequency; Fe is the estimated cumulative frequency; sup_x is the supremum difference; Y_i e $\hat{y}_i$ is the observed and estimated variables, respectively; $\bar{y}$ is the average of observed variable; n is the number of observations; p is the number of parameters.

In summary, the proposed methodology consisted of quantifying forest fragments given a desired interval of ecological indexes by using a pdf. The same procedure was done in the bivariate case, assuming as variables the area size and one of the indexes.

Thus, it is expected that this study may contribute to management plans of networks of forest seeds located in Brazilian Atlantic Forest, especially the "Forest Seeds Network of Surrounding the Caparaó and of Itapemirim River Basin". Among the contributions, the quantification and selection of well-conserved forest fragments were mentioned with the aim to collect seeds in forest fragments.

RESULTS AND DISCUSSION

Forest fragments (1,620) were identified and delimited corresponding to 924.11 km² or 13.7% of forest cover (Figure 2). The largest fragment found has area approximately equals to 43 km².

Functions for quantifying forest fragments

Area size

Table 2 presents the pdf parameters and statistically analyzes estimated frequencies to each area size group. The adjustments resulted in regression parameters and F values are significant at 95% probability level.

Weibull-3P pdf showed good results according to the statistics, in which the standard error of estimate in percentage (SEE%) ranges approximately from 12 to 29%. The adjusted coefficients of determination (R^2_{adj}) resulted in values larger than 0.91.

The Kolmogorov-Smirnov test revealed satisfactory estimates of the Weibull-3P pdf to the three groups. Figure 3 shows the observed and estimated frequencies of fragments, to the three area size groups.

From all observed fragments, an amount of 1,438

Table 2. Parameters and statistics of Weibull-3P pdf fitted by area size groups of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

Group	Parameters			Statistical analyzes			
	a	b	c	SEE (%)	$R^2_{adj.}$	F	KS
10-100 ha	10.9094*	18.4998*	0.8437*	12.258	0.9969	1,672*	0.010^{ns}
100-500 ha	110.1597*	122.2787*	0.7139*	18.085	0.9882	442*	0.013^{ns}
>500 ha	654.3558*	1,329.5343*	0.7892*	29.361	0.9157	43*	0.036^{ns}

*Significant at 95% probability level; ns = not significant.

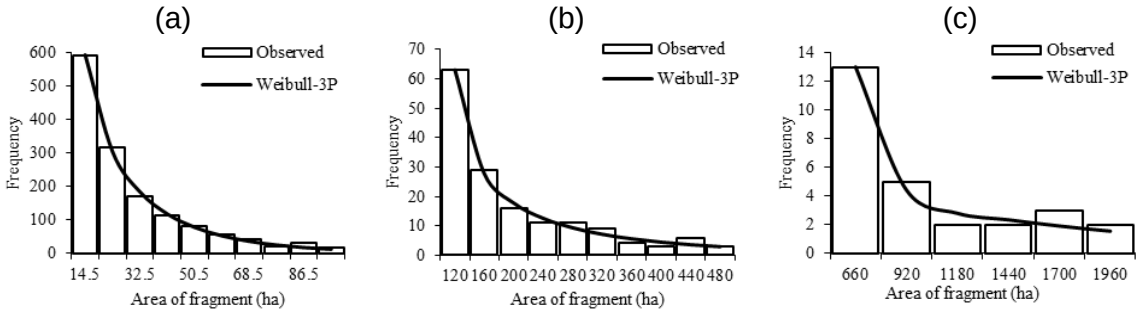

Figure 3. Frequency of forest fragments by area size groups from 10 to 100 ha (a), 100 to 500 ha (b), and larger than 500 ha (c), in the Itapemirim river basin and surrounding the Caparaó National Park.

Table 3. Parameters and statistics of the Weibull-3P pdf fitted to ecological indexes of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

Index	Parameters			Statistical analyzes			
	a	b	C	SEE (%)	$R^2_{adj.}$	F	KS
Fractal dimension	0.9936*	0.1306*	2.4321*	5.49	0.9954	1,132*	0.010^{ns}
Edge	0.0737*	0.4398*	2.7153*	4.47	0.9958	1,238*	0.015^{ns}

*Significant at probability level of 95%; ns: not significant.

(88.77%) has area between 10 and 100 ha, representing 417.34 km² of forest cover, whereas the fragments larger than 100 ha totalize 182 (11.24%), corresponding to about 600 km² of forest cover.

Although, fragments larger than 10 ha were mapped, Pirovani et al. (2014) also mapped forest fragments in part of our study area (Itapemirim sub-basin), finding around 68% of fragments smaller than 5 ha, and 22% between 5 and 50 ha. This means that if we would consider fragments smaller than 10 ha, we possibly would find most of the forest fragments in this class.

Ecological indexes

Table 3 shows the parameters and fit statistics of the estimated frequencies by the Weibull-3P pdf found to fractal dimension and edge index.

The coefficients and F test values were significant at 95% probability level and the KS values were not significant, indicating proper goodness of fit. The standard errors of estimate in percentage (SEE%) reached the maximum value of 5.5% and the adjusted coefficients of determination ($R^2_{adj.}$) were close to one.

Figure 4 shows the curve estimated by the Weibull-3P pdf and the observed frequency (Figure 4a), as well as the amplitude of the fractal dimension index to each area size group (Figure 4b).

The frequency distribution of the fractal dimension presented mode equal to 1.11 (Figure 4a), value interpreted as fragments with simple up to slightly irregular shapes (Hott et al., 2007). The index revealed a decrease of the amplitude with increase of group sizes, as well as the mean values tended to place in the middle of the amplitude. It is observed that the increase in area

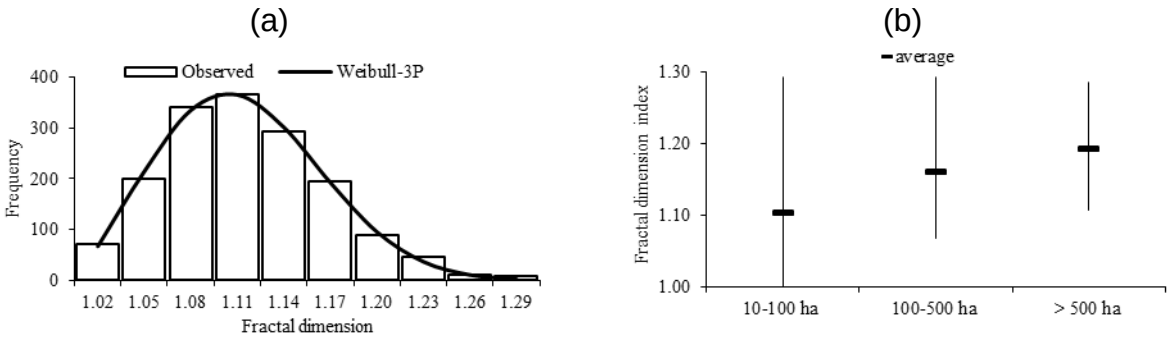

Figure 4. Frequency distribution (a) and amplitude (b) of fractal dimension index of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

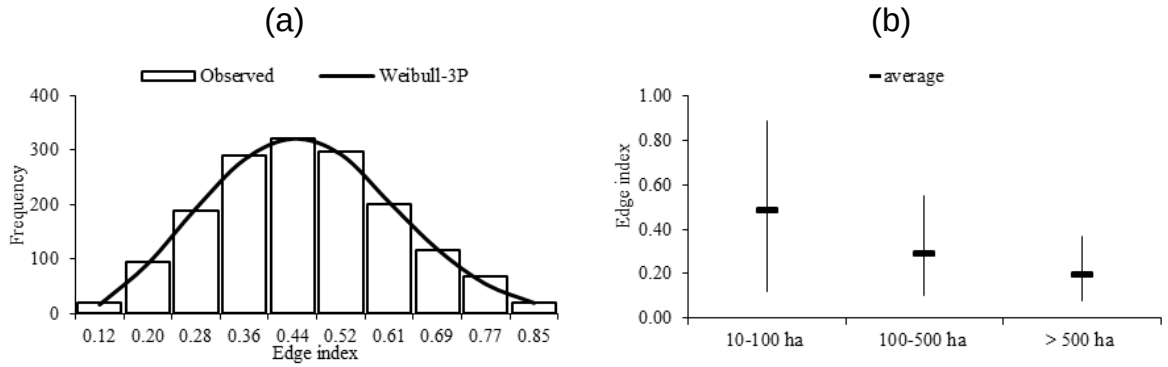

Figure 5. Frequency distribution (a) and amplitude (b) of edge index of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

size tended to increase the shape complexity of the forest fragments (Figure 4b).

Figure 5 shows the estimated curve by the Weibull-3P pdf and the observed frequency (Figure 5a) and the amplitude of edge index to each area size group (Figure 5b).

As observed in the fractal dimension index, the amplitude of edge index decreased with the increase in area size. The minimum, medium and maximum values to the group from 10 to 100 ha were equal to 0.12, 0.49 and 0.89, respectively. To the group from 100 to 500 ha, they were equal to 0.10, 0.29 and 0.55 respectively and, to the group of fragments larger than 500 ha, equal to 0.08, 0.20 and 0.37, respectively.

The fragments with rounded and simpler shapes occurred only in the group from 10 to 100 ha, while the fragments with area from 100 to 500 ha tend to have either elongated or very elongated shapes. Regarding to the fragments larger than 500 ha, the edge index (Figure 5b) and the fractal dimension index (Figure 4b) showed that they have very complex and elongated shapes. As consequence, these fragments have elevated perimeter-area ratio and are more susceptible to external factors (Laurance et al., 2000).

With respect to the normal bivariate functions, the initial hypothesis assumed to the Chi-square test was accepted, indicating data normality at 50% probability level. Therefore, its application was performed to each ecological index in relation to area size (ha) in logarithm scale. Table 4 shows the parameters μ_i and σ_i of the normal bivariate functions fitted to the three groups of area size (ha).

The integration of the bivariate functions (Equation 9) applied with the parameters (Table 4) allowed us to make3D graphs, which is as shown in Figure 6.

$$F(x_{1_a} \le x_1 \le x_{1_b}, x_{2_a} \le x_2 \le x_{2_b}) = \int_{x_{1_a}}^{x_{1_b}} \int_{x_{2_a}}^{x_{2_b}} \frac{1}{(2\pi)^{p/2}\sigma_1\sigma_2} e^{-\frac{1}{2}\left[\left(\frac{x_1-\mu_1}{\sigma_1}\right)^2 + \left(\frac{x_2-\mu_2}{\sigma_2}\right)^2\right]} dx_1, dx_2 \quad (9)$$

where x_1 is the ecological indexes; x_2 is the logarithm of area size (ha); x_{i_a} is the lower limit of the variable; x_{i_b} is the upper limit of the variable; σ_i and μ_i are the parameters of the model; p is the number of variables; *e* is the base of natural logarithm; π is the "pi" constant.

Table 4. Parameters of the normal bivariate functions applied to ecological indexes of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

Ecological indexes	Group	Parameters of x_1		Parameters of x_2	
		μ_1	σ_1	μ_2	σ_2
Fractal dimension index	10-100 ha	1.1033	0.0483	1.3821	0.2556
	100-500 ha	1.1604	0.0420	2.2451	0.1930
	>500 ha	1.1924	0.0375	2.9697	0.2262
Edge index	10-100 ha	0.4867	0.1426	1.3821	0.2556
	100-500 ha	0.2923	0.0859	2.2451	0.1930
	>500 ha	0.1983	0.0619	2.9697	0.2262

x_1: Ecological indexes; x_2: logarithm of area size (ha).

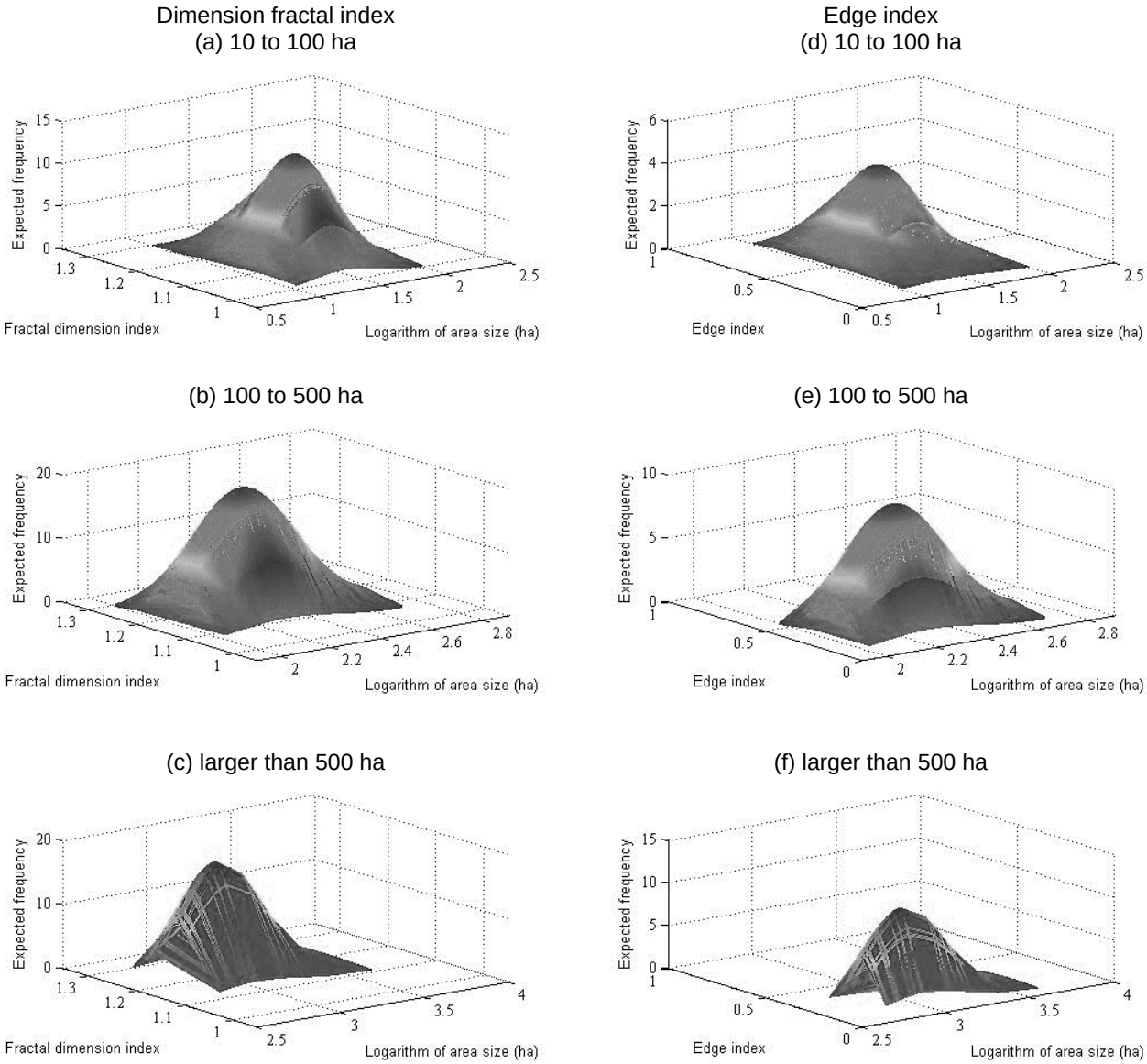

Figure 6. Normal bivariate distribution of the fractal dimension (a, b, and c) and edge indexes (d, e, and f) to groups of area size of forest fragments, in the Itapemirim river basin and surrounding the Caparaó National Park.

Table 5. Estimated frequency by normal bivariate functions for forest fragments larger than 500 ha, in the Itapemirim river basin and surrounding the Caparaó National Park.

Classes of area size (ha)		660-920	920-1,180	1,180-1,440	1,440-1,700	1,700-1,960	≥1,960	Total
Classes of fractal dimension index	1.01-1.03	-	-	-	-	-	-	-
	1.03-1.06	-	-	-	-	-	-	-
	1.06-1.09	-	-	-	-	-	-	-
	1.09-1.12	-	-	-	1	-	-	1
	1.12-1.15	3	-	-	-	-	-	3
	1.15-1.18	2	1	2	-	-	-	5
	1.18-1.21	7	-	-	-	2	2	11
	1.21-1.24	1	3	-	-	-	-	4
	1.24-1.27	-	1	-	1	-	-	2
	1.27-1.30	-	-	-	-	1	-	1
	Total	13	5	2	2	3	2	27
Classes of edge index	0.08-0.16	1	4	-	1	1	2	9
	0.16-0.24	9	1	2	-	2	-	14
	0.24-0.32	3	-	-	-	-	-	3
	0.32-0.40	-	-	-	1	-	-	1
	0.40-0.48	-	-	-	-	-	-	-
	0.48-0.56	-	-	-	-	-	-	-
	0.56-0.64	-	-	-	-	-	-	-
	0.64-0.73	-	-	-	-	-	-	-
	0.73-0.81	-	-	-	-	-	-	-
	0.81-0.89	-	-	-	-	-	-	-
	Total	13	5	2	2	3	2	27

Figure 6 shows graphs that represent the frequency distribution of forest fragments in bivariate case, where area size is represented by the x axis, ecological index by the y axis, and expected frequency by the z axis. The values of mode seen at the top of the distribution indicate a trend in increasing the fractal dimension index as the area size increases. The opposite trend occurred in the edge index, where the frequency decreases from larger fragments to smaller ones.

It can also be observed that the increase of the area size of the fragments makes the distribution to be more restricted, indicating that smaller fragments present largest values of fractal dimension and edge index, in which such variation reduces with the increasing of their area size.

Diagnosis of forest conservation status

The results allow us to consider that the studied area is too fragmented, reducing the number of fragments in the extent to which the forest area increases. Area size of the forest fragment is highly related to its capacity for inhabiting the biodiversity, which makes this variable very important for selecting well-conserved forest fragments (Santos et al., 2016; Viana and Pinheiro, 1998). This fact, also described in the island biogeography, was confirmed by studies that evaluated the relation between forest area and the number of species, such as Rush and Stutchbury (2008), Echeverría et al. (2007) and Nour et al. (1997).

Table 5 shows the frequency of forest fragments estimated by the normal bivariate function (Equation 9) for fragments larger than 500 ha, since they are more important in regards to forest conservation.

Despite the fact that the studied region has a mountainous relief, coffee monoculture is the dominant agricultural activity in the region (Mannigel, 2008; Sales et al., 2013; Pirovani, 2010). The influence of relief (slope, slope orientation, and altitude) on the spatial distribution of forest cover was studied by Santos et al. (2016a) and Silva et al. (2007).

In the surrounding Caparaó National Park, Santos et al. (2016a) found that the forest coverage in permanent preservation areas is strongly related to altitude, in which the greater human intervention occurs in altitudes below 1,110 m. In addition, Santos et al. (2016a) detected that the slope orientation directed to south and slopes larger than 45 degrees are the most forested areas.

Silva et al. (2007) also obtained relation with the presence of forest cover and slopes larger than 10 degrees and altitudes above 923 m, however, the authors found no influence of slope orientation.

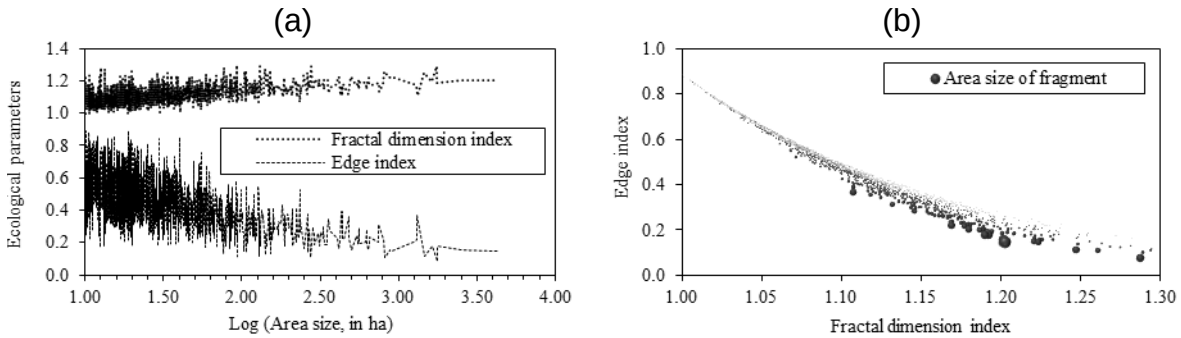

Figure 7. Relation between landscape ecology metrics of forest fragments in the Itapemirim river basin and surrounding the Caparaó National Park.

Thus, in relation to fractal dimension index, the lowest values induce us to associate with intense anthropic action basically by agricultural activities; on the other hand, the highest values indicate well-conserved fragments and low anthropic impact (Couto, 2004). Fragments with large area size generally are conservation areas protected by buffer zones that favor their surrounding preservation, allowing natural formations that promote increasing of shape complexity.

The small fragments in turn, generally are legal reserves of small and medium farms, oftentimes surrounded by grasslands, crops, and planted forests, which mischaracterizes the natural neighboring areas and reduces its fractal dimension index. As discussed earlier, steeper and higher altitude areas tend to present less agricultural activities than flatter and lower ones, thus, the more unfavorable is the relief, the more conserved the forest fragments tend to be (Silva et al., 2007).

In relation to edge index, according to limits established by Viana and Pinheiro (1998), approximately 80% of the fragments is very elongated (value less than 0.6), 18% is elongated (value between 0.6 and 0.8) and 2% is rounded (value greater than 0.8). Similar proportions were found by Nascimento et al. (2006) in forest fragments in the Itapemirim river sub-basin.

These results show the importance of maintaining buffer zones to the larger forest fragments, which favors the decrease of pressures on the biodiversity, while the smaller fragments act as stepping stones between the largest ones, contributing to the flow of animals and genetic interactions (Forman and Godron, 1981).

Figure 7a shows the variation of both indexes in function of the area size (in logarithm to the base 10). Figure 7b shows the scattering between the three landscape ecologic metrics, where the area size of the forest fragment is equivalent to the bubble size. The smaller and bigger bubbles are compatible to 10 and 4,300 ha, respectively. Figure 7a shows that the variation of the indexes reduces with the increase of the area size of the fragment. Especially in fragments smaller than 100 ha, such variation indicates the existence of different surrounding areas.

Such finding corroborates the existence of many forest fragments in different environmental conditions and edge effect, as described in Santos et al. (2016b), Harper et al. (2004) and Steininger et al. (2001). This suggest us that area size must not be the only criteria for selection well-conserved forests, since fractal dimension and edge index range even in fragments of equal areas.

We noted a clear trend in decreasing the edge index as the fractal dimension index increases (Figure 7a). Figure 7b indicates that the smallest fragments are well distributed along the both axis, on the other hand, the largest fragments are within a little more restrict interval of fractal dimension and edge index.

According to Figure 7, the forest fragments with area size larger than 500 ha are characterized by having complex shapes (large fractal dimension index), what provokes increases in the edge effect. However, such behavior also may occur in smaller fragments.

Santos et al. (2016) selected forest fragments to collect seeds employing other ecologic variables besides the ones used in this research, as internal area of forest fragment, distance among neighboring fragments, besides area size of neighboring fragments. The author observed that the size of the fragment is highly relevant for its selection if the objective is to collect seeds.

Echeverría et al. (2007) also employed similar indexes to evaluate impacts of forest fragmentation on species composition and forest structure. The authors observed that area size of fragment exerted strong effect in both components.

Conclusions

The largest forest fragments (>500 ha) differ from the smallest ones (<100 ha) in relation to ecological indexes. Larger forest fragments have more complex shapes and high edge effect. Smaller forest fragments have since simple shapes up to more complex ones, besides having

quite variable edge effect.

The frequency of fragments by area size presents an exponential model, consequently, the frequency of forest fragments decreases as much as its area increases. However, the fractal dimension and edge indexes follow a Gaussian model, thus, fragments with medium values for such indexes are majority in the region. Area size of the fragment, when modeled together with fractal dimension or edge index (that is, a bivariate case), have frequency following a Gaussian model, meaning that forest fragments with bigger areas, lower edge effects, and more rounded are the scantiest ones in the region.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Assessment of post-harvest losses of *Warqe* food products along the supply chain in Central Ethiopia

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Post-harvest food loss is a global problem but it is more critical in the food-insecure countries. Food waste occurs at different levels in the food supply chain from production through post-harvest handling to consumption. The presented paper aims at an assessment of post-harvest food loss of *warqe* foods along the supply chain and identified hot spots of the losses in the chain. *Warqe* (*Ensete ventricosum* (Welw) Cheesman) is banana like plant and it used as a food and non-food applications. *Kocho* and *bulla* are two main food products of the *warqe* plant. A total of 522 responders were randomly selected across the supply chain started from the two major *warqe* growing areas. *Kocho* and *bulla* reach final consumers through various channels. About 45.3% of *kocho* and 45.6% of *bulla* were lost from the total marketed product along the supply chain. The highest *kocho* (24.0%) and *bulla* (28.8%) losses were observed at retailer and processor levels, respectively. Practicing poor processing method, using perishable packaging material, poor transportation and inappropriate storage and market conditions were the main reasons for the losses. It is, therefore, important to work on value addition in *warqe* foods, improve processing storage and packaging technologies to reduce post-harvest losses.

Key words: *Bulla*, Ethiopia, *Ensete ventricosum*, kocho, post-harvest loss.

INTRODUCTION

Post-harvest food loss is a global problem but it is more critical in food-insecure countries. The Food and Agriculture Organization of the United Nations (FAO) estimated that one-third of food produced for human consumption is wasted globally, which is equivalent to about 1.3 billion tons per year. This loss leads to significant losses of resource used for food production (FAO, 2011). Post-harvest loss exists throughout the supply chain, from initial agricultural production down to final household consumption (Parfitt et al., 2010). Losses of food from farm to table through storage, transport, processing, and retail and in consumption are huge and associated water loss is significant, and therefore, reducing food loss and wastage reduces water needs in agriculture (Lundqvist et al., 2008).

In order to assess the magnitude of the food waste problem, it is first essential to define what food waste means. Losses of food are described using many

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different terms and there is no clear definition or demarcation between the terms *food losses, food waste,* and *post-harvest losses*. These terms are used in inconsistent ways in the literature and sometimes overlap. It is not easy to find one definition that combines all kinds of food waste. According to Grolleaud (2002), food loss is the loss in a quantity which leads the food to become unfit for human consumption. Food loss and waste is defined by Lipinski et al. (2013) in depth as "*the edible parts of plants and animals produced or harvested for human consumption but not ultimately consumed by people. It represents a decrease in the mass, caloric, and/or nutritional value of edible food intended for human consumption at any stage in the food value chain.*" According to (FAO, 2013), food loss implies the decrease in the quantity or quality of food which was originally intended for human consumption. However, food waste is defined as food appropriate for human consumption being a waste, whether or not it is kept beyond its expiry or left to spoil. The word food wastage includes both food loss and food waste together and it refers to any food lost by deterioration or waste. Food waste can occur at different points of food supply chain (Parfitt et al., 2010) and this wastage has a significant influence on the sub-Saharan African situation of food shortage (Affognon et al., 2015). It is believed that reducing of wastage of food by improving post-harvest management is more crucial than the production of more food by extensive agriculture horizontal expansion. Hodges et al. (2011) have also pointed out reducing food losses can increase food availability without requiring additional production resources and it can contribute to rural development and poverty reduction in less developed countries.

The main causes of post-harvest loss in low-income countries may include improper methods of harvesting, insufficient cooling, and unhygienic handling, lack of infrastructure, poor technical and managerial skill in food production and post-harvest (FAO, 2011). Bio-deterioration by microorganisms, insects, rodents or birds are also the main causes of postharvest loss in low-income countries (Hodges et al., 2011). Post-harvest loss ranges from about 21% for cereals up to 66% for fruits and vegetables in Sub-Sahara-African food value chains. Studies conducted in Ethiopia on seven fruit and vegetable crops (tomato, cabbage, onion, potato, mango, banana and avocado) revealed that the total average post-harvest losses range from 14 to 60%. Highest losses observed on cabbage were 58.9% and lowest loss (14.1%) recorded on onion crop (Gebresenbet et al., 2016). Developed world (North America, Oceania, and Europe) and the industrialized Asian nations such as China, Japan, and South Korea share about 56% of food loss and waste occurs from total loss whereas the developing world accounts for 44% of the loss (FAO, 2011). When looking at the distribution of the loss and waste, there is significant variation between developed and developing regions. In the developed countries loss occurs more at the consumption level, however, in developing countries, it occurs more during production and handling and storage (Lipinski et al., 2013). For instance, in Tanzania, maize losses that occur in the field are more economically significance than those which occur during any other single activity from harvesting to marketing (Abass et al., 2014). Main causes of significant post-harvest losses in the early stage of the supply chain in developing countries are financial and structural limitations in harvest techniques, storage, and transport infrastructures, combined with climatic conditions favourable to food spoilage (FAO, 2013).

Relatively there are high food losses of fresh root and tubers in developing countries early in the supply chain (FAO, 2011). As a result wastage of starchy roots appears in the top 10 because of high wastage volumes in the agricultural and post-harvest phases (FAO, 2013). One reason for this high loss in these countries is the perishable nature of the crops which make them easily damaged during harvesting and post-harvest activities especially in the warm and humid climates (FAO, 2011). However, many of the developing countries peoples are highly dependent on root and tuber crops as a source of food, nutrition and cash income (Scott et al., 2000). In Ethiopian root and tuber crops are the third national food commodity next to maize and wheat in the quantity of production (CountrySTAT Ethiopia, 2016). Cassava, potato, sweet potato, and yam are the major root and tuber crops in worldwide and reported that about 50 millions of hectares occupied by these crop in 1995-95 worldwide (Scott et al., 2000). Some other root and tuber crops are very specific to a certain country, for instance, *warqe* is a staple food in Ethiopia.

Warqe (*Ensete ventricosum* (Welw.) Cheesman) is banana like plant and sometimes it calls "*enset*". *Warqe*-based farming plays a significant role in food security of Ethiopia (Brandt et al., 1997). According to Gebremariam (1996), *warqe* grows in Sub-Saharan countries and Asia mainly as a wild plant and only domesticated in Ethiopia. The complex *warqe* farming system is the most sustainable indigenous farming activity in Southern and South-western Ethiopia and supports the densely populated highlands of these regions in terms of food sources (Tsegaye and Struik, 2002). Ethiopian economy as a whole strongly depends on agriculture. In 2016, Ethiopian population estimated about 92.2 million (ECSA, 2016) and over 84% of the population lives in rural areas, where crop production and animal husbandry are their main livelihoods (CountrySTAT Ethiopia, 2016b). Agriculture accounts for 42.9% of gross domestic product (GDP) of the country (MoFED, 2014) and it contributes to nearly 80% of export earnings. Moreover, it provides employment opportunity to 73% of the population (EATA, 2014). The exact number of people that depend on *warqe* food is not known. Thus, based on the 2014 Central Statistical Agency of Ethiopia agricultural sample survey report (ECSA, 2014) and 2012 population projection

(ECSA, 2013), it could be estimated that about 35% of Ethiopians, living in the *warqe* production areas, use *warqe* as their staple food. In addition, *warqe* is commonly used as food in big cities like Addis Ababa, Awassa, Dilla, Adama, Jimma, Sodo, Hosaena, Wolkite, Woliso, Bonga, Arba Minch, and other cities, which makes that more than 35% of the Ethiopian population consuming *warqe* as food.

Production of *warqe* foods is processed by traditional knowledge, using locally made traditional tools (Hunduma and Ashenafi, 2011). The major food products of the *warqe* plant are *kocho, bulla* and *amicho*. *Kocho* is the dough which is the bulk of the fermented starch obtained from the mixture of the decorticated leaf sheaths and pulverized corm. *kocho* processing involved a two-stage fermentation process and fermentation takes place in earth pit. After two or three months, fully fermented *kocho* can be produced (Tuffa, 2016). The bread that is prepared from fermented *warqe* is also called *kocho*-bread. It is common to serve in the restaurant's menu *kocho*-bread with *kitfo* (*kitfo* is traditional Ethiopian food which is prepared from chopped red meat mixed with spiced butter). *Bulla* is white dry powder or semi-liquid which is produced by squeezing the decorticated leaf sheaths and pulverized corm and decanting the liquid. It is eaten as porridge and dumpling. *Amicho* is non-fermented the corm of *warqe*, which is consumed after boiling just like other root and tuber crops (Brandt et al., 1997).

Mogessie and Yewelsew (1996) reported that about 33% of *kocho* spoilage happens during storage. Traditional *kocho* fermentation and extended period of storage in the pit has shown spoilage problem and it creates bad smell (Brandt et al., 1997; Hunduma, 2012). Hunduma (2012) indicated that works done on the post-fermentation loss of *warqe* primary food products are very minimal and no scientific attempts have been made to improve storage facilities for products. Even though *warqe* has multiple uses, the production, and processing of this "valuable" crop is poorly investigated. Unlike many other crops, *warqe* has had very little research conducted on it. Very limited information about the post-harvest loss of *warqe* primary food products is available. Moreover, the cause of spoilage is not clearly known so far and the extent of loss in the supply chain of *warqe* has also not been studied and documented. Thus, the main objective of the present study was to assess the post-harvest losses of *warqe* food products along the supply chain and to identify hot spots of the losses in the chain. The specific objectives were to quantify post-harvest losses of *warqe* food products at different chain levels and to identify the factors responsible for the losses.

MATERIALS AND METHODS

Selection of study area

The study was conducted during 2014 across the major *warqe* growing areas of West Shoa and Southwest Shoa Zones of Oromia Region, Ethiopia. Two major *warqe* growing areas namely Haro Wanchi and Maruf were purposely selected for this study. These study sites were selected with the consultation of relevant agricultural officials and based on preliminary survey result made in 2013. The result of preliminary indicated that Haro Wanchi and Maruf areas are main sources of *kocho* and *bulla* supplier to the central market (Addis Ababa, the capital city of Ethiopia) (Tuffa, 2016). Haro *kocho* market, Haro open market, Haroj and Woliso *kocho* main market were selected by following *kocho* and *bulla* supply in Haro Wanchi area. Guder Odo-Bari *kocho* open market and Guder *bulla* market were selected by following supply from Maruf area. All these markets are feeders to Addis Ababa Merkato *kocho* market, which was also included in the study. To collect data regarding *bulla* processing in Addis Ababa and Woliso cities were selected. Consumer related information was collected from Haro Wanchi, Guder, Ambo and Addis Ababa.

Sample size and sampling technique

Multistage sampling procedures were used to select representative respondents from the study areas. In the first stage, *wraqe* grower households from Haro Wanchi and Maruf areas were selected. Then traders, transport operators, small-scale food processing enterprises and consumers were selected by flowing *wraqe* products supplied from the two growing areas. Appropriate sample sizes were determined using Equation 1, assuming that there is no significant difference in population of *warqe* grower farmers (Yamane, 1967):

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

Where n is designated as the sample size the researcher uses, N is designated as total number of households in Haro Wanchi (680) and Maruf (525) areas; N = 1205 *warqe* growing household in total, *e* is designated as maximum variability or margin of error 0.063 and 1 designates the probability of the event occurring. Thus samples of *warqe* grower households were determined as 209.

For transport operators, small-scale *bulla* processing enterprises, and cultural restaurants, we considered all the aforementioned in the area because of their small population. Generally, in analysing the supply chain and post-harvest loss of *warqe* foods a total of 522 respondents were selected by using simple random sampling method for collecting primary data and information at production, processing, distribution and consumption levels. Among the total respondents interviewed, 209 were *warqe* growing households, of which 91 households from Maruf area and 118 households from Haro Wanchi were selected.

A total of 56 *kocho* and *bulla* traders were interviewed in all marketplaces. About 15 respondents were selected from *kocho* and *bulla* transport operators. Eight traditional food processing enterprises were included in this survey. Interviews were also held with *warqe* consumers both at household and restaurant levels for assessing post-harvest losses of *warqe*. A total of 223 *warqe* foods consumers at household level were randomly selected and interviewed. Again, 11 cultural restaurants were purposively selected from Addis Ababa city and included in the study.

Method of data collection

Five sets of pre-tested semi-structured interview questionnaires translated to the local language were used for collecting data and information from all respondents. Data collections were done by eight data collectors out of them two were researchers and six were data enumerators. Trained enumerators were used with close supervision and involvement in data collection by the researchers to

avoid variation between data collectors. These respondents comprised of *warqe* growers, processors, traders (intermediaries), transporters and consumers. The data collected were reviewed and the information required to address the specific aspects of the study was extracted and utilised. Secondary data were gathered from the agricultural offices of the respective districts, journal articles, and other research reports. Market information was received from tax offices and market owners of respective *kocho* and *bulla* markets.

Socio-economic characteristics of the farmer such as family size, the age of the farmer, education level, land ownership, total farm size and *warqe* farm size were considered and recorded.

Method of estimation of different post-harvest food losses

Post-harvest food losses in this study refer to measuring quantitative and qualitative losses that occurred at each level in the supply chain. Quantitative loss implies the loss of physical substance of these products which are reflected in weight loss. The quality loses in this study is expressed in a change of colour, taste, and odour in *kocho*, *bulla,* and their food products. However, the qualitative loss is more difficult to measure because of the lack of quality criteria that are easily measurable.

The post-harvest loss was assessed by adopting LaGra (1990) Commodity System Assessment Methodology (CSAM). CSAM is made up of 26 components in four subsections that together account for all the steps associated with the pre-production, production, post-harvest handling and marketing of any given commodity. CSAM helps to quantify the losses and identify the causes of losses at different points of the food supply chain. Field data from different respondents (farmers, transporters, traders, food processors, and consumers) were collected on quantity basis and post-harvest losses obtained at different operations and different levels. *Warqe* growers were asked through questionnaire-based interview what quantity of *kocho* and/or *bulla* they produced during 2014. To assess post-harvest losses, farmers were asked how much quantity of *kocho* and/or *bulla* products was lost during each operation (harvesting, sorting, processing, fermentation, storage, and transporting to market).

Traders' level losses were estimated as the quantity of the *warqe* product lost during trading in the same period. During interviewing, the traders were asked what quantity of *kocho* and/or *bulla* was bought and sold. Then the losses at different levels of traders (transportation, handling etc.) were estimated in terms of the quantity bought.

Losses at consumers' level were estimated on the basis of the quantity lost at households and restaurants. Post-harvest losses were also estimated for different types of losses such as weight loss, rotten or spoilage, and physical losses, etc. The characteristics and symptoms of different types of losses were explained to the respondents and it helped to identify and quantifying the losses they experienced. Then, the individual losses were calculated in reference to the total quantity of the *warqe* product and expressed in percentage. For the calculation of total losses in terms of percentage, it should be noted that the total cannot be taken as the sum of the percentages at each loss stage. Thus, if the producer losses, wholesaler losses, food processing losses, retailer losses and consumer losses were x1, x2, and x3, ...,xn, then total losses was calculated as:

$$x1+ (100 – x1)\times x2/100+ [100-(100-x1)\times x2/100] \times x3/100 +\ldots$$

Data analysis

Data analysis was made using MS Excel and IBM SPSS Statistics software version 22. Using the pivot table and figure, depending on the type of data, means, standard deviation and/or frequencies were computed. T-test, least significant difference (LSD) test was used to identify significant differences between *warqe* growing areas and value chain stage (P<0.05) at 95% confidence interval level.

RESULTS

Warqe production

Warqe production is one of the main farming activities in the areas of Maruf and Haro Wanchi. As shown in Table 1, the size of farmland and a number of *warqe* stand in the farm significantly differed between Maruf and Haro Wanchi areas. Average farm land size per household in Maruf (1.93 ha) was larger than the Haro Wanchi (1.01 ha) area. The size of farm land covered by *warqe* in Maruf area was larger (3,413.19 m^2) as compared to Haro Wanchi (1,955.35 m^2). However, land covered by *warqe* was not significantly different between the two areas. On the other hand, the number of *warqe* plants per household in Haro Wanchi area (688 plants) was higher as compared to Maruf area (226 plants).

The maturity stage of *warqe* plant differs from plant to plant and it depends on the *warqe* cultivar planted and climatic conditions of the area. The present study has shown that *warqe* plant in Haro Wanchi matures (6.07 years) earlier than that of Maruf area (4.57 years). However, *kocho* yield per plant in Maruf was higher (64.82 kg/plant) as compared to Haro Wanchi (29.08 kg/plant) and this yield positively correlates with a number of plant per unit area (Table 1). Under the condition where the number of *warqe* plant per unit area is small there is a higher yield of *kocho* per plant. With regards to *bulla* and *amicho* yield per plant, there is no significant difference between the two study areas. A highly significant difference in the length of *kocho*, *bulla,* and *amicho* storage period is observed between Haro Wanchi and Maruf. *Kocho* and *bulla* on average stores for 302.74 and 262.68 days in Maruf, respectively; but in Haro Wanchi *kocho* can be stored on average for 90.75 days and *bulla* for 35.11 days. *Kocho* and *bulla* can store for a longer period of time compared to *amicho*. It was observed that in Maruf the maximum storage time for *kocho* was 730 days while it was for 540 days for *bulla* whereas in Haro Wanchi *kocho* stores for 365 days and *bulla* for 180 days.

Warqe production is the main source of household income in the study areas (Table 1). There was a highly significant difference in income generated from *warqe* in the two areas. On average, a household can get up to3,106 Ethiopian Birr per year (1 US dollar ≈ 21 Ethiopian Birr) in Haro Wanchi, whereas in Maruf a household can get about 1,336 Ethiopian Birr per year from warqe sale. The study also has revealed that Maruf farmers travel more distance (8.5 km) to sell their warqe products as compared to Haro Wanchi area (3.86 km).

Table 1. Farm size, land cover by Warqe, maturity time, yield and storage time, contribution of Warqe to income in year and distance to market in the study areas (mean ± SE).

Parameter	Area		Mean
	Maruf	Haro Wanchi	
Size of farm land (hectares)**	1.9 (±0.2)	1.0 (±0.4)	1. 5 (±0.1)
Land covered by *warqe* plant (m^2)ns	3413.2 (±739.2)	1955.3 (±685.4)	2900 (±538.7)
Numbers of *warqe* plants in a farm**	225.9 (±13.3)	687.7(±33.3)	408.5 (±24.9)
Warqe plant mature (year) **	4.6 (±0.1)	6.1 (±0.1)	5.4 (±0.1)
Kocho yield (kg/plant) **	64.8 (±3.7)	29.1 (±0.8)	43.8 (±2.0)
Bulla yield (kg/plant)ns	4.4 (±0.5)	3.7 (±0.2)	3.9 (±0.2)
Amicho produce (kg/plant)ns	14.1 (±5.2)	17.1 (±1.3)	17.1 (±1.3)
Kocho stored (in days) **	302.7 (±15.4)	90.8 (±7.5)	185. 7 (±10.9)
Bulla stored (in days) **	262.7 (±18.2)	35.1 (±19.9)	112.4 (±16.8)
Amicho stored (in day) **	1.0 (±0.0)	1. 9 (±0.1)	1.9 (±0.1)
Revenue from *warqe* (Birr/year) **	1335.9 (±207.7)	3105.6 (±207.7)	2754.0 (±180.9)
Distance to marketplace (km) **	8.5 (±0.9)	3.9 (±0.1)	4.90 (±0.3)

**Highly significant difference (*P<0.01*) between *Warqe* growing areas (Maruf and Haro Wanchi) at 95% confidence interval of the difference; ns non-significant difference between *Warqe* growing areas at 95% confidence interval of the difference.

Purpose and uses of *warqe* growing

The survey result indicates that *warqe* plant is the major food and non-food crop in the study areas. Almost all respondents (94%) of *warqe* growing farmers noted that *warqe* makes an integral part of their livelihood and 6% of them consider as less important for their livelihood. All respondent farmers mentioned that they mainly grow *warqe* for multipurpose. The purpose of *warqe* crop production in the study areas is presented in Figure 1. *Warqe* growers were putting in the order of importance *warqe* crop production for family livelihood as food, construction purpose, leaves used for bread making, a plant used as soil conservation, to make household utilities, for income generating, medicinal value for human and animal, feeding the animal, compost, and fuel.

Supply chain of *warqe* foods

The supply chain of *warqe* food products is illustrated in Figure 2. Producers, collectors, wholesalers, retailers, processors, transporters, open market dealers and consumers (households and restaurants) were the main actors in *kocho* and *bulla* supply chain. As described, Chaka et al. (2016) producers are referred to *warqe* growing farmers. Collectors are non-licensed traders who operate in the primary market (local market). They are one of the basic key players in the local market. The collectors generally run their business with wholesalers. They buy *warqe* products directly from producers in the vicinity of growers and sometimes at the local market and transport to the marketplace and then sell all the collected products in a large amount to wholesalers at the local market.

Wholesalers are big traders and generally operate between rural market and urban market. They have fixed establishment in the marketplace with a short time storage facility. They purchase a lot of *warqe* products from producers or through collectors and sell a large amount of *warqe* products to retailers and large consumers like restaurants. The retailers have permanent shops or places in the market. In the shops, they have a storage place. They purchase products in a bulk amount from their suppliers and sell in a small amount to their clients. *Bulla* processors are those who are involved in the processing of *bulla*. They purchase fresh *bulla* in a large amount and then process it into dried products and sell in bulk or in a small quantity to their customers. Transporters are those who participate in the transporting of *warqe* products by using vehicles from local market to central market or somewhere from market to processing place and from processing place to market. Open market dealers are retailers and have a permanent place in the urban market. They purchase a few products from retailers or processors. They run their business with other commodities and sell their products to the consumer at urban market. Consumers are final consumers and those who make food from *kocho* and *bulla* for direct use by themselves or to sell the foods which are prepared from *warqe* products to their customers in the restaurants.

It was observed that the supply chain is long and often overlapping. The relationship of *warqe* supply chain actors is complex. Producers sell their products either to wholesalers, collectors and/or to consumers. The proportion of the amount sold depends on the availability of buyers and nearness of market. Collectors purchase a

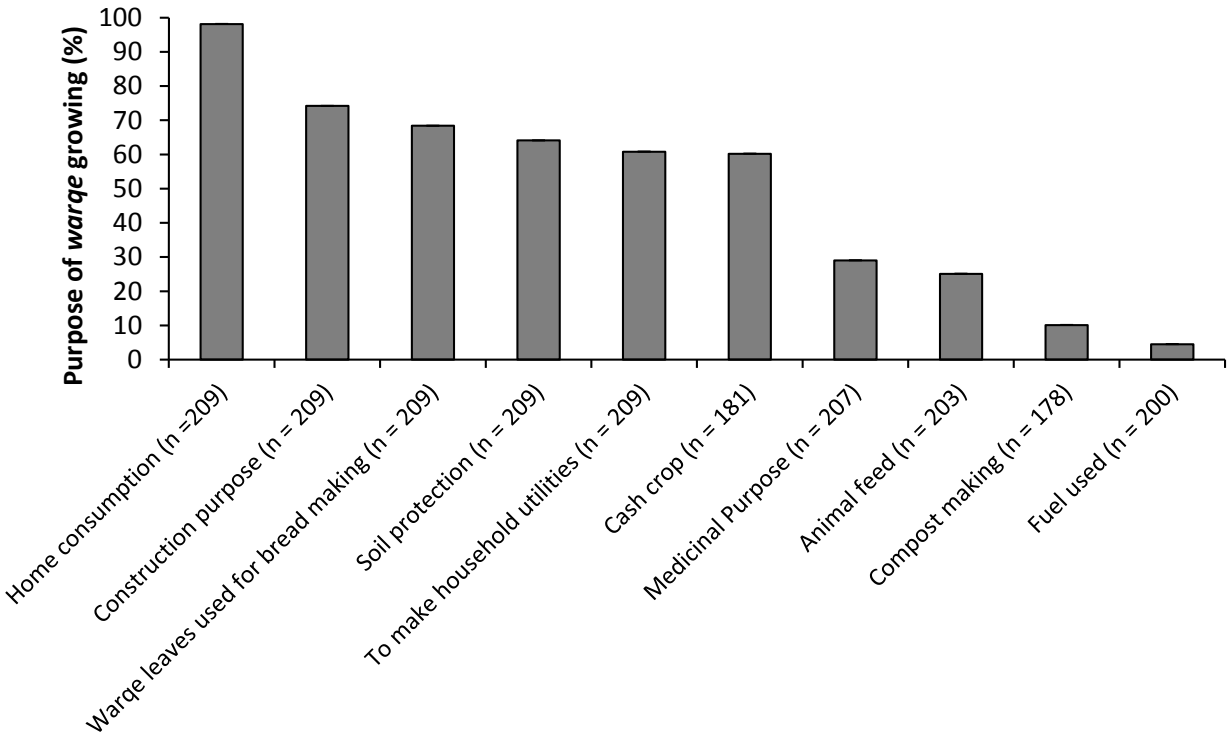

Figure 1. The multiple purposes of warqe crop production in the study areas (n is the number of respondents).

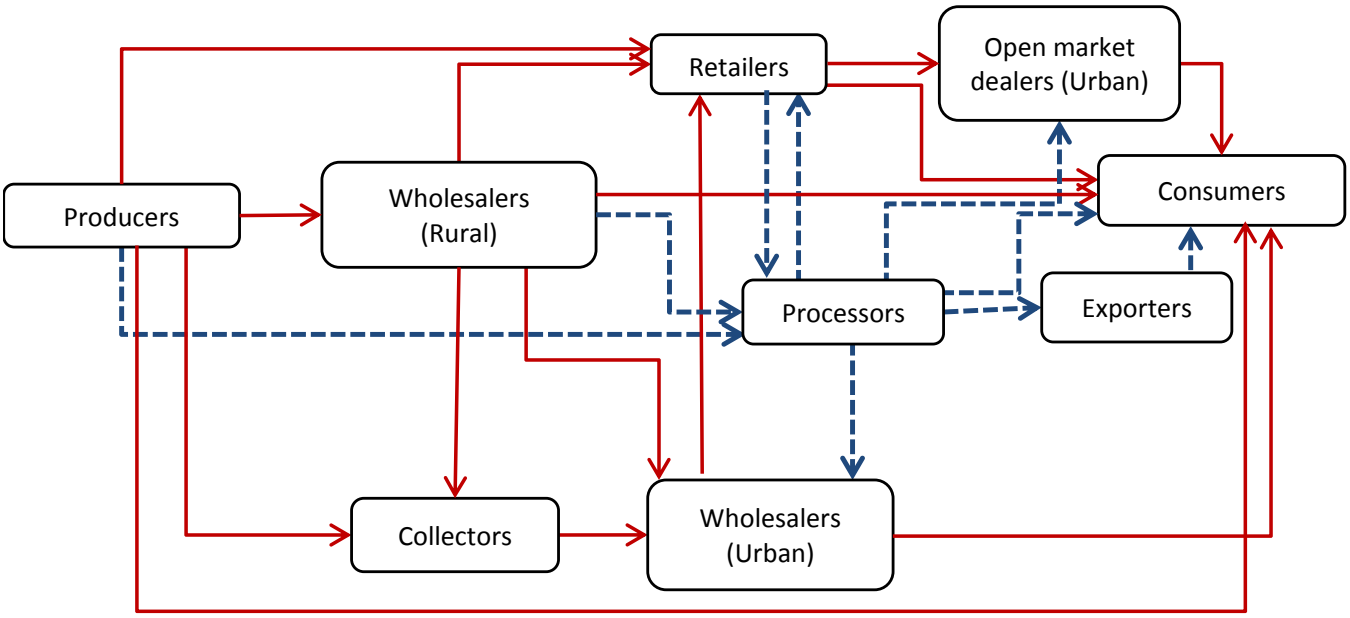

Figure 2. Schematic mapping of the *kocho* and *bulla* supply chain in the central part of Ethiopia. Red solid arrows indicate that physical flow of *kocho* and fresh *bulla*. Blue dash arrows indicate the physical flow of processed *bulla*.

large amount of *kocho* and fresh *bulla* from producers and wholesaler in the vicinity of farmers and at the local market. They sell directly to urban wholesalers. Wholesalers buy *kocho* and fresh *bulla* from producers and then sell to urban wholesalers, retailers, and consumers. Urban wholesalers sell their *kocho* and fresh

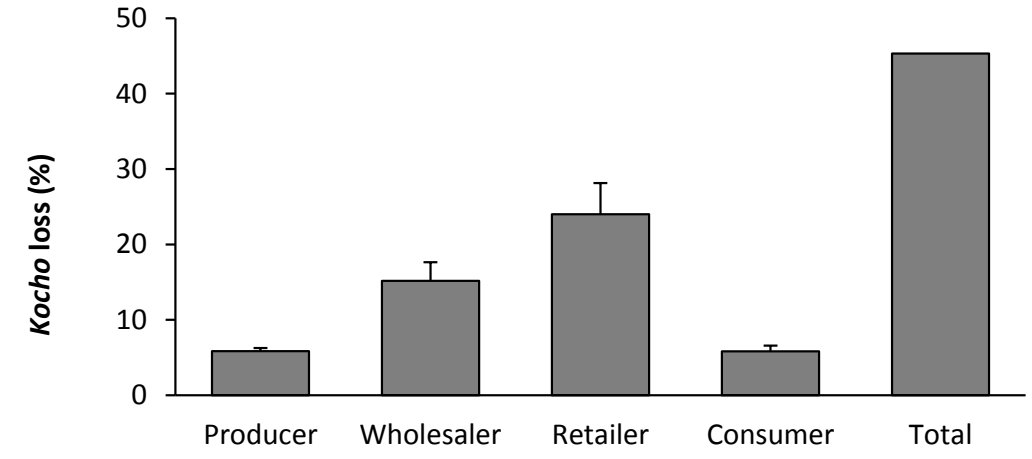

Figure 3. *Kocho* loss (%) at different stages of warqe supply chain in Central part of Ethiopia (mean ± S.E).

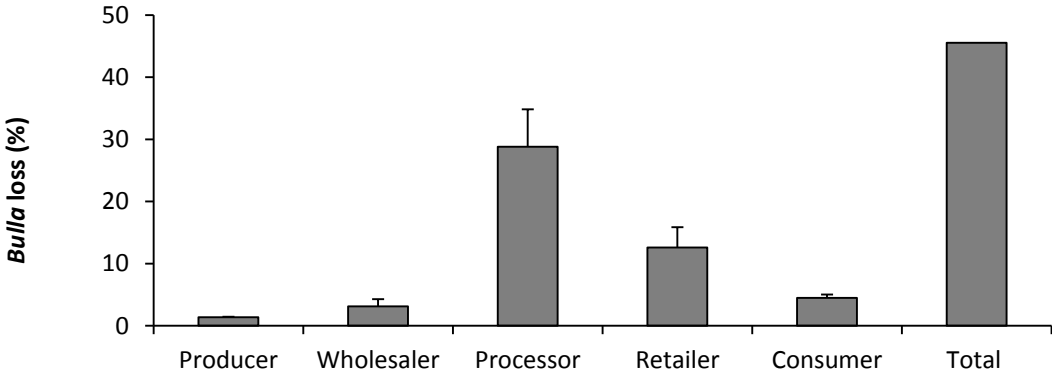

Figure 4. *Bulla* loss (%) at different stages of warqe supply chain in Central part of Ethiopia (mean ± S.E).

bulla to retailers and directly to consumers. Retailers buy from wholesalers and sell larger portions, to consumers and the rest to open market dealers in urban areas. Open market dealers buy from retailers and directly sell to consumers.

It observed that the supply chain of *bulla* is different than that of *kocho*. In *bulla* supply chain, both fresh and processed *bulla* products are involved. The supply chain of fresh *bulla* is similar to that of *kocho* as described above. For the purpose of processing, fresh *bulla* is purchased by processors from three different suppliers which include: Producers, wholesalers, and retailers. Processed *bulla* is mostly sold to wholesalers, retailers and open market dealers and some amount directly to the final consumers. Very small amount of processed *bulla* is sold to exporters. Exporters export the products to different countries mainly to Ethiopian traditional restaurants and shops which are found abroad. Thus, final consumers can get *kocho*, fresh and processed *bulla* from different suppliers through a number of chains.

Post-harvest food losses in the supply chain

Substantial losses were observed in the whole supply chain of *warqe* food products. There was a highly significant (P<0.01) difference in the extent of loss between the chain actors for both *kocho* and *bulla* (Figures 3 and 4). It was calculated that about 45.3% of *kocho* and 45.6% of *bulla* are lost from the total marketed product of *kocho* and *bulla* in the supply chain respectively. In *kocho* supply chain, the highest loss is estimated at retailer levels. For *kocho*, the loss at retailer levels is 24.0% of the total loss of the same product at all supply chain levels. Figure 5 shows the spoiled *kocho* at marketplace due to damage by rodents (A) and poor display and exposure to the air (B). The lowest *kocho* losses were estimated at producer and consumer levels to have been about 5.8%. Similarly, in the *bulla* supply chain, the highest loss (28.8%) is reported at processor level (Figure 4). The lowest loss is observed in *bulla* at producer level which is about 1.4% of the total *bulla*

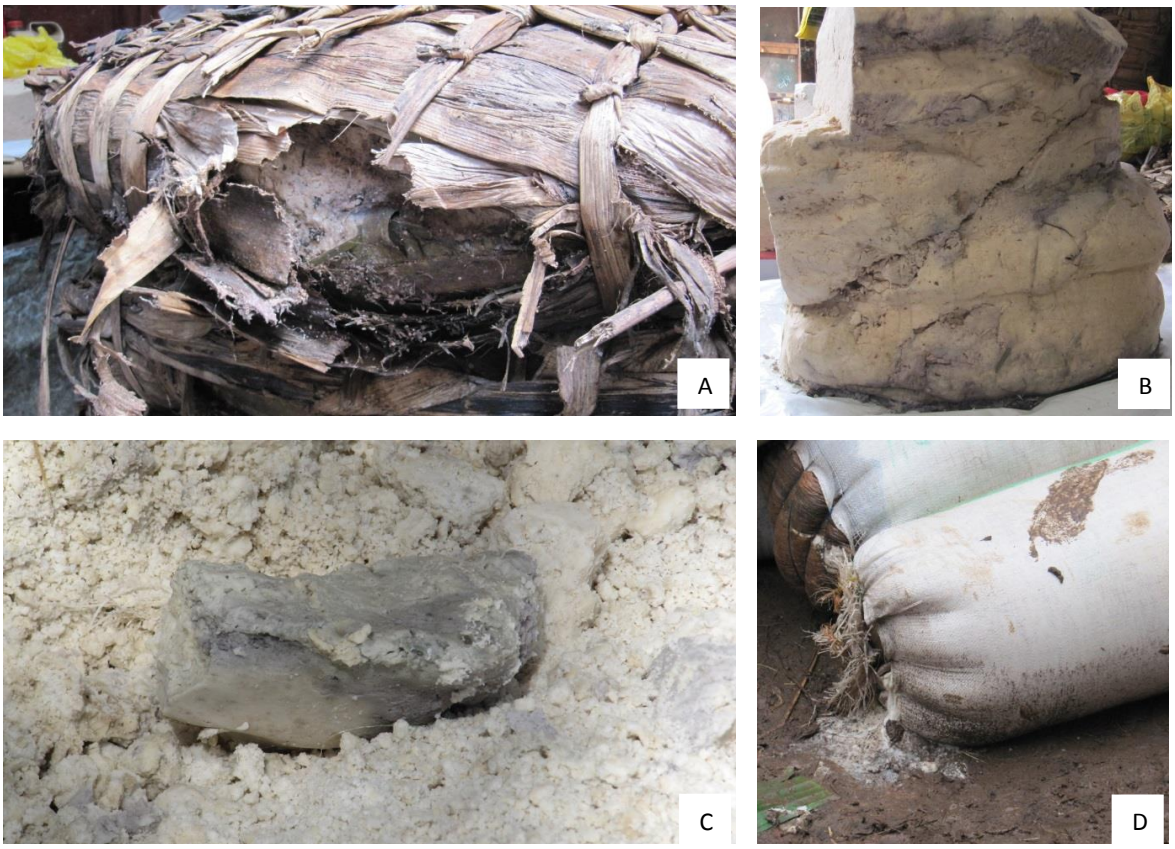

Figure 5. Spoiled *kocho* at marketplace due to damage by rodents (A) and poor display and exposure to the air (B). *Bulla* spoilage at processing place due to mould development (C) and physical loss due to poor packaging (D).

produced. In general, the losses of *bulla* and *kocho* are similar in the chains.

Loss of *kocho* and *bulla* at producer level in the study areas is shown in Figure 6. Loss of *bulla* is reported to be found only during storage time at the producer level. No loss was reported for both *kocho* and *bulla* during harvesting and transportation. However, some physical losses of *warqe* are observed while harvesting, but it was difficult to quantify that losses. There was highly significant difference (P=0.000) in the extent of loss reported of *kocho* in the storage at Maruf and Haro Wanchi areas. There was also a highly significant difference (P=0.002) in the extent of loss reported of *kocho* during fermentation process between Maruf and Haro Wanchi areas. However, there was no significant difference in the extent of *bulla* losses between the two areas (P=0.106). The highest loss of *kocho* has been reported at Maruf area both in the storage (6.7%) and during the fermentation process (5.8%). The lower losses have been reported at Haro Wanchi, which was about 3% loss during fermentation and 1.6% loss in the storage. Main causes of losses during the fermentation process of *kocho* were the entrance of flood water during the rainy season to the fermentation pit, exposure of products to air and sunlight, improper decorticating process, mould development and soil contamination. Rodent attack (mainly mole rats), the insects (termites and ants) damage has been specifically reported in Maruf area, mould development and physical loss were causes of loss in the storage.

Fermentation and storage losses are higher in Maruf area as compared to Haro Wanchi area. The reason of higher loss observed in storage at Maruf area is due to the fact that *kocho* is usually stored for a long time of period at Maruf (303 days) whereas at Haro Wanchi *kocho* is stored on average for 91 days (Table 1). The main reason of higher loss occurs in fermentation period at Maruf is reported that there are higher termite and ant pests existing in the area. These insects live in the fermentation pit and cause spoilage of *kocho*.

There are the highest losses of *kocho* and *bulla* recorded at trader level. The losses recorded at wholesalers and retailer level is shown in Figure 7. There was a significantly different (P=0.016) in storage loss of *kocho* observed between wholesaler and retailer levels. The loss during transportation to the marketplace is observed only at the wholesale level. However, the loss reported during *kocho* and *bulla* processing and that of *bulla* at storage is not significantly differed between wholesalers and retailer level. The loss at the storage

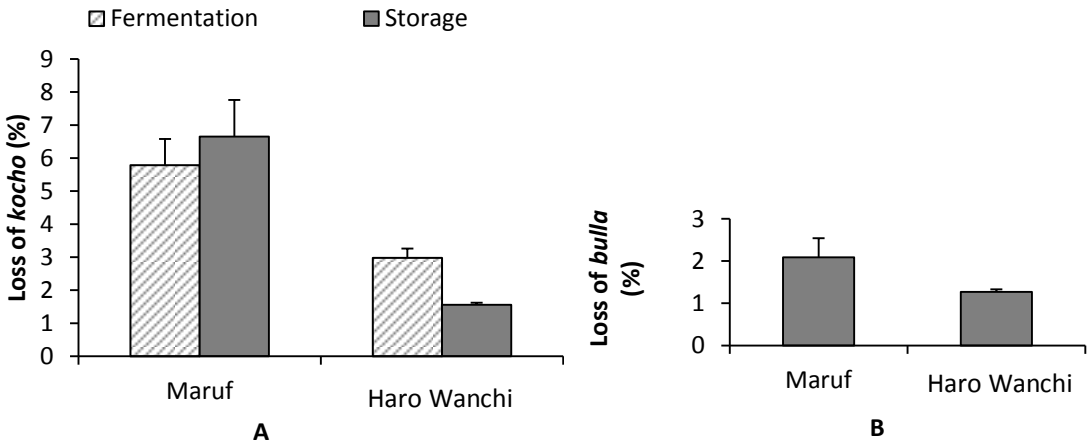

Figure 6. Loss of *kocho* (A) and *bulla* (B) in percentage at producer level (mean ± SE) from the total produced.

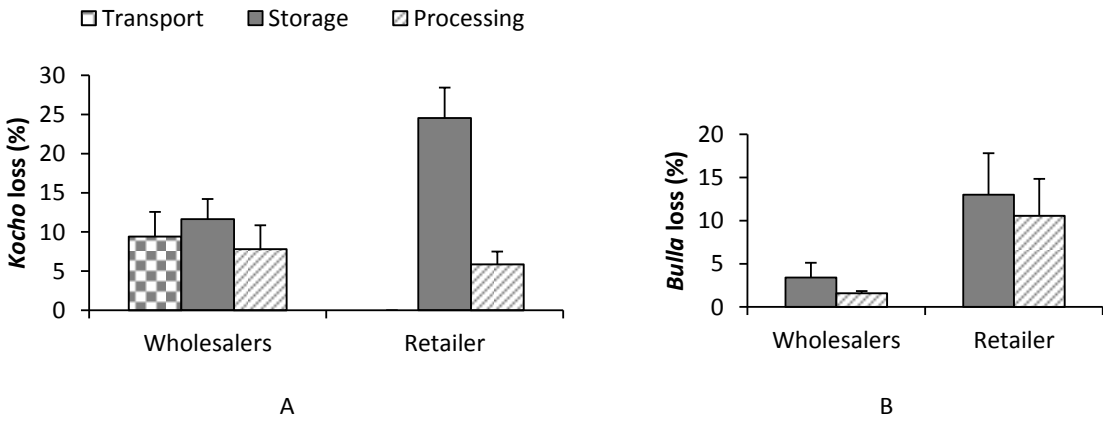

Figure 7. Estimated loss of *Kocho* (A) and *bulla* (B) at wholesalers and retailer levels (mean value + SE) from total purchased.

Table 2. *Kocho* and *bulla* storage duration and distances of suppliers to marketplace at trader level (mean value ± S.E).

Parameter	Business group			Mean
	Wholesalers	Collectors	Retailer	
Distances of suppliers travel to market (Km) *	30.92 (±3.87)	20.00 (±2.89)	62.25 (±13.55)	41.95 (±5.32)
Kocho stored (days)**	8.26 (±1.62)	7.00 (±0.00)	29.35 (±4.65)	16.12 (±2.32)
Bulla stored (days)ns	113.53 (±54.30)	7.00 (±0.00)	98.67 (±29.77)	97.79 (±28.04)

** Highly significant difference (*P<0.01*) between business groups at 95% confidence interval of the difference'; * Significant difference (*P<0.05*) between business groups at 95% confidence interval of the difference; ns non-significant difference between business groups at 95% confidence interval of the difference.

both for *kocho* and *bulla* shows the most remarkable loss at trader level. Storage losses in *kocho* (24.5%) and that of a *bulla* (13.0%) from total purchased products at the retailer are the highest losses recorded. The main factors responsible for losses during transport to the marketplace are poor packaging, leakage (physical loss) and poor transporting methods. The main factors for the storage loss in both *bulla* and *kocho* are reported to be poor packaging, long period storage (Table 2) and mould development. The cause of *kocho* and *bulla* losses at market place processing is mainly due to lack of suitable processing place.

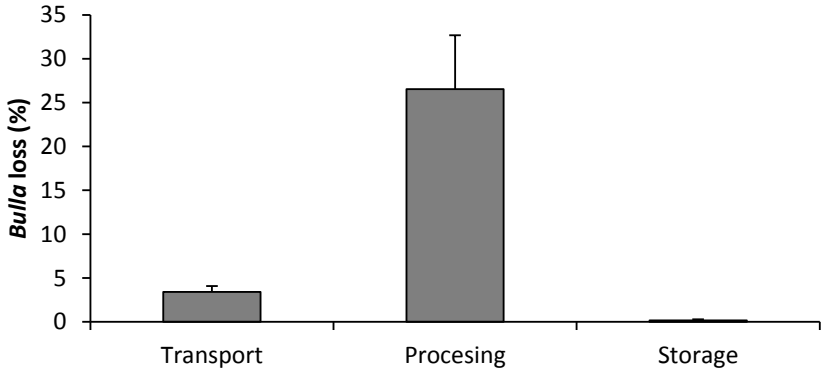

Figure 8. Loss of *bulla* experienced by the processor (mean value + SE) from the total purchased *bulla*.

Table 3. *Kocho* and *bulla* purchased annually (mean ± S.E) and storage (day ± S.E) at consumer level.

Parameter	Types of consumers			Mean
	Household in countryside	Restaurant	Household in city	
Warqe foods purchased in a year (Kg) **	759.7 (±58.2)	22934.5 (±10460.3)	21.9 (±6.6)	1770.9 (±571.6)
Kocho stored (days) **	195.20 (±10.85)	31.91 (±8.20)	130.50 (±80.03)	185.46 (±10.49)
Fresh *bulla* stored (days) ns	104.16 (±11.15)		103.63 (±41.99)	104.14 (±10.78)
Dried *bulla* stored (days)			105.45 (±29.50)	105.45 (±29.50

**Highly significant difference (*P<0.01*) between types of consumers at 95% confidence interval of the difference; ns non-significant difference between types of consumers at 95% confidence interval of the difference.

Bulla loss at processor level

There is high loss observed during *bulla* processing (Figure 8). Highly significant differences (*P*=0.001) are observed between the losses that occur during its transportation to processing points, during processing operation and storage at processing points losses. The highest *bulla* loss is observed at processor level which is about 26.5% of the total fresh *bulla* purchased. The lowest *bulla* loss was the storage loss (0.2%). The main reasons for loss at transport and storage were poor package and poor transport handling which result in a physical loss. Poor quality products for processing and physical loss during cleaning, washing, and drying activities were the main reasons for losses at processing operation. The development of mould and deterioration in quality due to exposure to air was another cause of loss at the processor level.

Warqe foods loss and wastage at consumer level

From *kocho* and *bulla*, different kinds of food are prepared. *Kocho*-bread, *Qummusi* (bread made from *kocho* mixed with cereals flour), *Honkuroo* (it is prepared from best quality of *kocho* mixed with spiced butter served for a highly respected guest); porridge and soup are some of the food types prepared from *warqe* in the study areas. The amount of consumption varies depending on the types of consumers (Table 3). On average, about 760 kg of *warqe* food is consumed per year in the countryside at household level with an average family size of 6.3. However, in the city at a household level, about 22 kg of *warqe* foods per year is consumed. *Kocho* is mainly consumed both at household and restaurant levels whereas *bulla* is mainly consumed at the household level.

At the consumer level, there was a significant amount of food wastage observed (Figure 9). There were highly significant differences (*P*=0.000) on loss observed between different kinds of losses at the consumer level. Highest food wastage was observed due to leftover food (3.7%) from total prepared food, however, no significant difference in the loss that occurs with *kocho* in storage (3.4%) from total purchased at the consumer level. The lowest loss was observed at storage of fresh *bulla* (1.2%). Food wastage due to the leftover was mainly because of the excess or over-preparation of food, improper preparation of food and excess provision of food. Major causes of loss of *kocho* in storage at consumer level were its exposure to the air and contamination of soil in the storage pit. Physical loss, sticky nature of *bulla* and inappropriate food preparation were the main reasons for the loss during food

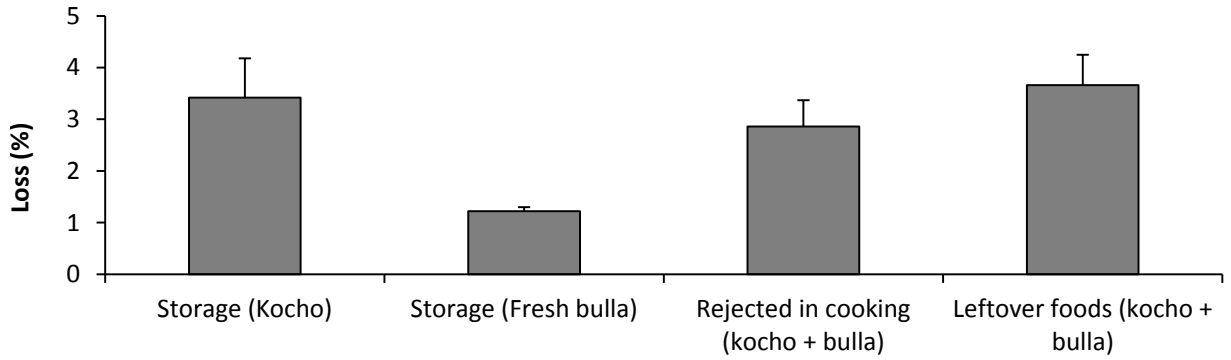

Figure 9. Warqe foods loss and wastage at the consumer level (mean ± S.E).

preparation of *bulla*.

DISCUSSION

Warqe production is unique as compared to cereal crops and needs complex processing to produce *kocho* and *bulla*. The farmers describe that in the study areas at least one *warqe* seedling needs to be three times transplanted to the permanent site. One fully mature *warqe* plant needs on average six to seven years after final transplanting to permanent planting site to reach harvesting. To get fully fermented, *kocho* needs two to three months fermentation process in the pit. Farm and processing activities are gender-based labour division. It is observed that unlike other crops all post-harvest and trading activities of *warqe* is mainly done by women. Moreover, men are not involved in the marketing activities even in the central market in Addis Ababa; most of the *warqe* traders are women. In the study of Abass et al. (2014) indicated that in Tanzania maize processing activities mainly done by women. In line with our observation, Chiche (2012) and MacEntee et al. (2013) reported that women as a sole responsible body in decision making for processing; marketing and in *warqe* income controlling at the producer level. The role of men and women in *warqe* cultivation and processing are clearly distinguished. Women have the responsibility to secure household food by transforming the *warqe* plant into *warqe* food (Negash and Niehof, 2004). This indicates that tradition and cultural influence of *warqe* production and trading are very unique and it is considered as taboo if men are involved in the processing and trading activities. It makes the burden on women, as they are responsible for every post-harvest activity and this cultural tradition makes life very tough for women in the *warqe* growing areas throughout the country.

Growers use inputs like cattle manure as organic fertilizer and planting materials for *warqe* production from their own source. This indicates that in the *warqe* production system there are no external farm inputs suppliers. Brandt et al. (1997); indicate that *warqe* farming mechanisms allow long-term high productivity with minimum external inputs. However, there are some research findings and experiences which indicate that there are improved *warqe* varieties and inorganic fertilizer application technologies developed by research centres. However, these technologies are not communicated to the farmers in the study areas. This implies that farmers do not have other options to use improved technologies like planting materials and fertilizer as a result of which they kept on using their own sources of farm inputs.

The results of the present study show that *warqe* is grown in the study areas for multipurpose uses (Figure 1). Farmers expressed that *warqe* is everything for their livelihood; it is food, it is feed to animals, medicine, packaging material, plates, beds, construction materials to build house and fence and even a source of water for cattle during the dry season. *Warqe* is also household's main income source and the majority of farmers (38%) responded that more than 75% of their household income comes from *warqe* production. This implies that *warqe* production is a very crucial farm activity for their family livelihood. In agreement with this study, previous study reports of the show that the livelihood of families in the *warqe* producing areas depends on *warqe* (Brandt et al., 1997; Negash and Niehof, 2004; Degu, 2012; Teamir and Tilahun, 2012). *Warqe* production is the basis of household food security (Negash and Niehof, 2004); it is insurance against hunger (Brandt et al., 1997) and more than just a food crop grown for multipurpose (Brandt et al., 1997; Degu, 2012; Teamir and Tilahun, 2012). It also has a potential of being used as industrial raw material to produced fibre-related goods and starch for paper and adhesives (Brandt et al., 1997; Bezuneh, 2012).

Most of the past studies were conducted in the southern parts of the country and reported about multiple uses of *warqe* focusing on family food security. However, the present study shows that *warqe* is used as a cash crop in addition to other benefits of this crop. This could be due to two main reasons: The first reason could be that the study areas have very suitable climate to grow *warqe* plant and this gives the best quality product with higher yields. *Warqe* product of the study areas is known

for its quality and it is known by a brand name “Yechebo *kocho*” in the market. The second reason is that the areas are located very close to the central market (Addis Ababa Merkato market) and thus the *warqe* products of these areas highly demanded in the market. These all indicate that *warqe* grown in the areas is not only for food but also enables farmers to get motivated by non-food products and multiple uses for their livelihood, including its use as a source of income.

It has been observed that *warqe* foods supply chain is long and complex (Figure 2). *Kocho* and *bulla* reach to final consumers through different suppliers in complex chains. According to Khatami et al. (2015), a supply chain defines networks among suppliers, manufacturers, transporters, warehouses, retailers and customers, which are systematized to transform raw materials into finished products and allocate the final products among customer through retailers. However, in the present study, supply chain refers to the sequential arrangement of different chain actors involved in the movement of *warqe* food products from producers to ultimate consumers. In the chain of *warqe* food products, mainly *kocho* and *bulla* follow from producers to final consumers through numbers of chain actors.

Generally, *kocho* and *bulla* reach the consumers in various ways. Rural consumers directly get from producers and wholesalers in local marketplaces. However, urban consumers in cities access through retailers, processors and open market dealers. The supply chain of processed *bulla* is different from the supply chain of the fresh *bulla*. In line with our study report, Degu (2012) also reports that similar ways of the supply chain are reported for fresh *bulla* in the Southern part of Ethiopia. There are multiple causes for the complexity of *warqe* food product supply chain including the producers and wholesalers in rural areas do not have the information about central markets, the transportation and market facilities are poor, there is an absence of links between producers and retailers, processors and consumers, and a lack of cooperation among producers. This is compounded by the nature of products like bulkiness and easy perishability. In addition, there is a lack of support from government to *warqe* markets, dominance of central market suppliers by a few people and difficulty for new players to enter central markets.

A significant amount of *warqe* foods is wasted throughout the supply chain, from initial *warqe* growing down to final household and restaurant consumption (Figures 3 and 4). The results show that highest loss occurs with *kocho* at retailer and *bulla* at processor levels while the lowest loss is recorded at the producer and consumer levels in both food types. In a report of FAO (2011) it is indicated that generally, food loss mainly occur at early and middle levels of food supply chain and less food is wasted at the consumer level in the low-income countries. Similar to the current study result FAO (2011) report shows that highest losses occur in post-harvest handling and storage and processing and packaging, and the lowest losses are observed at consumption for roots and tubers in sub-Saharan Africa. This indicates that *warqe* food loss has the same trends of losses that occur in roots and tubers in the sub-Saharan Africa.

Highest *kocho* (24.0%) and *bulla* (12.6%) losses are observed at retailer levels particularly market storage. These highest losses reflect that the *warqe* food products are traded in poor hygienic conditions in the market. Traders sell the products by displaying at open air. There is no proper storage place for products and traders use the same place for selling and storage. This condition is not suitable for selling and storage. The markets are very crowded and not have a good ventilation system. It is observed that *kocho* and *bulla* are handled roughly during loading and unloading and even stacking in the stored place. Packaging material for *kocho*, which is a wrapping by *warqe* leaves at local markets, is not replaced until it reaches final consumers. The leaves become dry and deteriorate when they reach the central market because of rough handling during transport and due to delicate nature of leaves. Moreover, to check the quality of the products, there is a practice by traders piercing the packaging leaves to take out samples in the marketing chain. This opened hole gives a chance of exposing the product to air and, even flies have access to lay their eggs and eventual larvae can develop in storage time. All these stresses have their contributions to the deterioration of *kocho* at the retailer level. These may be causes for highest *kocho* and *bulla* losses in retailer levels. In *bulla* supply chain the highest loss is observed at processing levels (28.8%). One of the causes of high food loss in developing countries is a lack of processing facilities (FAO, 2011). The traditional method of processing, using inappropriate equipment, lack of quality products and a poor method of drying are the most probable reason for this highest processing loss of *bulla*.

At the producers level, the highest loss of *kocho* is found in storage as compared to the loss which occurs in fermentation. This may be due to the reason that *kocho* could be stored for long period of time in the pit. This long storage in the pit may cause higher loss of *kocho* due to contamination with soil, exposure to air and flood water. In both study areas, the loss of *bulla* is small as compared to a loss of *kocho*. This may be due to the following main reasons: unlike *kocho,* the nature of *bulla* is not easily spoiled, usually, *bulla* is not stored in the pit; the amount of production of *bulla* in very small in one harvesting time and producers gives much care to *bulla* because of its highest value in the market. In the work of Mogessie and Yewelsew (1996), it is reported that *kocho* loss reaches about 33% in storage. Hunduma (2012) points out *warqe* primary food products at producer level are serious and liable to loss. The loss of *warqe* food means the loss of investment which expends on the plant for long years through cultivation and processing

practices. In our study also, it is clearly indicated that *warqe* food loss is observed at the producer level. Therefore, *warqe* food loss in producer level is important and the loss is more pronounced in the storage and during formation processes.

The loss at trader level is a most significant source of loss in the *warqe* supply chain. Our result has clearly shown that there is the highest loss of *kocho* and *bulla* recorded in storage at retailer level (Figure 7). This highest loss of *kocho* at retailer occurs due to the long travel distance from suppliers and stored for long times (Table 2) as compared to wholesalers. In this long distance transportation, the products are exposed to stress like overloading, exposure to strong sunlight, air, and dust. Moreover, *kocho* is stored in the market in poor storage with poor handling practices. The method of *kocho* displaying in retailer marketplace are very poor and result in the exposure of products to air and light. One of the natures of *kocho* is its changing colour when exposed to air. The blackish colour is an indication of poor quality *kocho*. Inappropriate handling in the storage exposes the products to rodents (mainly rats) attack. Package material is damaged by rats, the result which leads to exposure to air and contamination with fungus and also contaminates the food directly with urine, faeces, and pathogens from rat feet and fur. As a result of this, colour changes and mould development are observed.

The loss during *bulla* processing, as has been revealed by this study is the most significant source of loss in the *bulla* supply chain. This highest loss happens during the main processing operation. Using of the poor and traditional method of processing may be the main cause of *bulla* wastage. Processors use inappropriate tools for processing like large barrels and sieves. Using of this kind of equipment may be increasing the loss. The way of drying *bulla* on open plastic sheet on the ground may cause to expose it to dust contamination and wind blowing. As a result, of these poor ways of drying method *bulla* physical loss and the discard of fresh *bulla* due to quality problems are observed. Lack of quality products for processing, a poor package of the fresh and processed *bulla*, poor transportation and handling are additional causes for this high loss. Similar to this study in cassava processing discarding due to small and woody tubers are reported to be the main causes of cassava processing loss (Oguntade, 2013). This indicates that one of the main causes of loss at processor level in the root and tuber including *warqe* is a lack of quality and suitable raw materials for processing.

Foods prepared from *kocho* are commonly consumed both at household and restaurant levels. However, foods prepared from *bulla* are commonly restricted to only household level. This may be the foods prepared from *bulla* are traditionally labelled as ‘female food’. At the consumer level, a remarkable amount of *warqe* foods is lost in the course of food preparation and home storage (Figure 9). Unlike other chain levels, the loss in consumer level is observed to be low in *bulla* supply chain (Figure 4). This might be due to a small amount of *bulla* purchased and given much care to the product in consumer level as compared to other levels of supply chain actors. Similar kind of result is obtained in potato post-harvest loss study in Bangladesh (Hossain and Miah, 2009). Losses which are estimated due to leftover and *kocho* storage show high losses when compared to another kind of losses at the consumer level. These may be due to the reason that foods prepared from *warqe* have short shelf-life and inappropriate handling food in the home. Traditionally, in the study areas food is served to people over the limits of the consumer. Thus, due to this custom, excess foods are prepared and leftover foods are common phenomena. This tradition may be main causes for food wastage at the consumer level.

Conclusion

This paper assessed post-harvest losses of *warqe* food products along the supply chain and identified hot spots of the losses in the chain. The result showed that there are eight supply chain actors involved in the *warqe* foods supply chains. *Warqe* growers, collectors, wholesalers, retailers, processors, transporters, open market dealers and consumers were identified as the main actors in the very complex supply chain of *warqe*. *Kocho* and *bulla* reach to consumers through various channels. Significant amounts of *warqe* foods were found to be wasted throughout the supply chain, from initial *warqe* growing down to the final consumer stage. The overall losses of marketed *kocho* and *bulla* were 45.3 and 45.6%, respectively, in the supply chain. The highest losses of *kocho* (24.0%) were observed at the retailer level and the highest losses of a *bulla* (28.8%) at the processor level. Lack of appropriate processing technology at producer and processor level, use of poor storage facilities, packaging materials and transport methods, poor handling in the market and air exposure during market display, insect pests at the producer level and rodent problems at farm and market level are the main causes of *kocho* and *bulla* losses. Therefore, it is important to work on value addition to *warqe* foods, improvement of processing technology, transportation, storage, packaging and handling, and improvement of market conditions to reduce post-harvest food losses of *warqe*.

Conflict of Interests

The authors have not declared any conflict of interests.

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