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African Journal of Biotechnology

Full Length Research Paper

Seasonal influence on sexual hormones and semen plasma parameters of Arabian sand gazelles (*Gazalla subgutrosa marica*) in Saudi Arabia

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The plasma levels of testosterone (T), follicular stimulating hormone (FSH) and luteinizing hormone (LH) were evaluated in Arabian sand gazelles *Gazalla subgutrosa marica* to reveal their relation with the sexual desire and testicular efficiency. The levels of those hormones fluctuated over the year; the FSH peak (2.212 ml/µlu) was in October (the beginning of reproductive season). LH level peaked in November (0.73 ml/µlu), while the testosterone level started to increase from July and reached its peak during September and October (340.38 and 419. 5 ng/dl, respectively) and decreased rapidly after November. The levels of some semen inorganic constituents were also measured, the average of sodium, potassium and chloride concentrations were 652.75 ± 124 ± 2.3 and 7.2 ± 0.15 ml mol/L, respectively. Fructose level averaged 141 ± 3.5 mg /dl. The use of the reproductive biotechnologies, which aim to preserve the endangered mammals including Gazelles, is restricted by several factors and depends upon the understanding of the relevant reproductive physiology. In conclusion, sexual hormones were strongly influenced by season. The results from this study suggest more investigation on the reproductive physiological research in order to detect the suitable period for semen collection of Arabian Sand Gazelles *(G. subgutturosa marica)* in Saudi Arabia.

Key words: Testosterone, follicular stimulating hormone (FSH), luteinizing hormone (LH), fructose and *Gazalla subgutrosa marica*.

INTRODUCTION

The Arabian Sand Gazelles *(Gazella subgutturosa marica)* is classified as vulnerable (VU) on the IUCN Red List (IUCN Red, 2009) and listed under appendix II of the convention on migratory species (CMS, 2005). Scientific references on the Arabian Sand Gazelles concern only the ethological aspects and reintroduction as well as veterinary issues and diseases (KKWRC, 2008). Yet, the information on reproductive physiology is still lacking. A few reports concern these issues (Al-Eissa and Alhomida, 1997; Al-Eissa, 2007a, 2007b) as the knowledge on reproductive male characteristics is crucial for protection of the particular species from extinction.

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Testosterone is a steroid hormone from the androgen group and is found in mammals, reptiles, birds, and other vertebrates. In mammals, testosterone is secreted primarily by the testicles of males. It is the principal male sex hormone and an anabolic steroid (Alder and Cox, 1983). Follicle-stimulating hormone (FSH) is a glycoprotein gonadotropin secreted by the anterior pituitary in response to gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus. The same pituitary cell also secretes luteinizing hormone (LH), another gonadotropin. FSH and LH are composed of alpha and beta subunits. The specific beta subunit confers the unique biologic activity. FSH and LH bind to receptors in the testis and ovary and regulate gonadal function by promoting sex steroid production and gametogenesis (Grover and Malm, 2005; Bassil and Morley, 2009). FSH stimulates testicular growth and enhances the production of an androgen-binding protein by the Sertoli cells, which are a component of the testicular tubule necessary for sustaining the maturing sperm cell. Hence, maturation of spermatozoa requires FSH and LH. Stimulation of LH estrogen and progesterone production from the ovary.

FSH and LH secretion are affected by a negative feedback from sex steroids. Inhibin also has a negative feedback on FSH selectively. High-dose testosterone or estrogen therapy suppresses FSH and LH. Primary gonadal failure in men and women leads to high levels of FSH and LH, except in selective destruction of testicular tubules with subsequent elevation of only FSH, as in Sertoli-cell-only syndrome. Previous study indicated that the whole reproductive system of fallow deer is directly controlled by the photoperiod and that related morphological and physiological processes are indirectly determined by photoperiodic changes (Rolef and Fisher, 1990).

Seasonal levels of LH, FSH, testosterone (T) and prolactin (PRL) were determined in plasma of adult male Southern Pudu deer (*Pudu puda*) kept in Concepcion, FSH concentrations remained at peak levels (54 to 63 ng/ml) from December to March; minimal values (25 to 33 ng/ml) were detected from April until October. Testosterone levels exhibited two, almost equal peaks; the first peak (2.8 ng/ml) was detected in March and the second one (2.7 ng/ml) in October. Both T peaks were preceded by an earlier elevation of LH in February and July (both around 1.3 ng/ml). During the fall, only the alpha male exhibited a sharp peak of T (8.4 ng/ml), whereas in the spring five out of six bucks demonstrated an increase of T levels. Two peaks of LH and T and the 4 months of elevated FSH may be related to a long period of spermatogenesis observed in this species (George et al., 1996). The role of the adrenocortical system in the regulation of plasma levels of reproductive hormones was investigated and plasma levels of LH, testosterone (T), FSH, prolactin (PRL) and androstenedione were determined in male white-tailed deer after ACTH, The result showed no effect on FSH and PRL levels (Bubenik et al., 1990).

MATERIALS AND METHODS

All of the experimental procedures were conducted in King Khalid Wildlife Research Center (KKWRC) (25°03'N, 46°45'E), Saudi Wildlife Commission (SWC), Riyadh, Saudi Arabia.

Animals

Ten (10) males of Arabian Gazelles *G. subgutrosa marica* aged 2 to 4 years (average 15 to 18 kg live weight each) served as serum donors. The males were kept in enclosure 100 x 100 m and all were good health well feed mainly on dry alfalfa, some concentrated pellets and water.

Radioimmunoassay (RIA) for hormones

Testosterone, concentrations in the serum was quantified by commercial available RIA kits (Immunotech, France). The levels of FSH, LH were measured by rate specific FSH and LH RIA kits (DRG international, Germany).

Semen collection and processing

Semen collection

Semen was collected from the males by using an electroejaculation procedure described by Al-Eissa (2007) and Al-Eissa et al. (2007; 2009) (Figure 1). The animals were anaesthetized using Xylazine (8.4±1.5 mg kg^{-1} b.wt.) and ketamine hydrochloride 6.9±1.5 mg kg^{-1} b.wt.). During the electroejaculation procedure, which typically involved <10 stimulations up to maxima of 4.5 to 7.0 volts, also similar method was described by Holt et al. (1988; 1996). Semen analysis was carried out immediately after collection. Seminal plasma sodium (Na), potassium (K) and chlorides (CL), were assessed with commercial kits (Labtest Diagnostica, Brazil) using an electrolyte analyzer (Iselab Eletrolyte Analyser, Brazil) to determine seminal plasma concentrations of each element. Qualitative measurement of fructose in seminal fluid was carried out by Resorcinol method (Fauser et al., 1990).

Statistical analysis

All data were expressed as mean±SD. The difference in serum concentration of FSH, LH, and Testosterone was evaluated by one-way ANOVA. Differences were considered to be significant at $p<0.05$.

RESULTS

The concentrations of serum levels of Testosterone T, FSH and LH were measured by Radioimmunoassay RIA method. The obtained results fluctuated throughout the year with high levels during mating season as shown in Table 1. The mean of FSH hormone was significantly ($p<0.001$) increased compared with other months and reached its peak (2.212 mμ) in October which is the

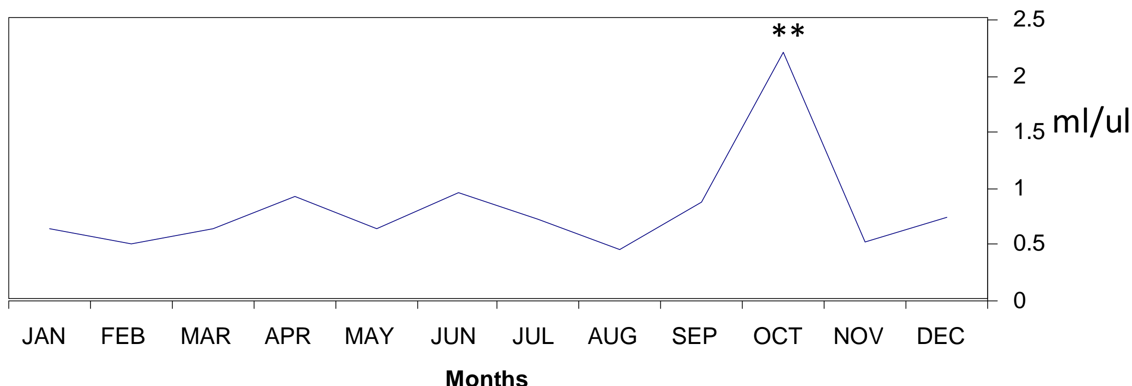

Figure 1. FSH concentration during a year.

Table 1. Hormones (Mean±SD) in males of Arabian Gazelles (*Gazalla subgutrosa marica*) through the year (sample volume 5 ml).

Month	(FSH) (ml/µlu)	(LH) (ml/µlu)	(T) (ng/dl)
Jan.	0.637±0.182	0.188±0.0125	34.75±6.58
Feb.	0.511±0.115	0.130±0.0153	39.20±10.7
Mar.	0.651±0.066	0.114±0.0143	34.29±12.14
Apr.	0.926±0.207	0.050±0.0129	36.22±4.88
May	0.641±0.130	0.078±0.005	66.75±18.8
Jun.	0.955±0.285	0.0613±0.009	45.86±4.08
Jul.	0.736±0.186	0.0700±0.00	158.63±39.53 *
Aug.	0.450±0.161	0.053±0.0115	226.0±55.11**
Sep.	0.870±0.066	0.325±0.313**	340.38±38.06**
Oct.	2.212±0.472**	0.217±0.0167	491.5±41.14**
Nov.	0.524±0.102	0. 73±0.003**	104.25±25.4
Dec.	0.741±0.124	0.200±0.00	117.5±10.83
Over mean	0.82± 0.14	0.184±0.025	141.3±41.8

Significant value ** p<0.001 and *p<0.01.

sexual season Figure 1. Also, LH hormone increased one month before FSH and significantly (p<0.001) increased compared with other months and reached its peak 0.73 mµ during September and November Figure 2. In contrast, testosterone hormones significantly (p<0.01) increased from May until August and significantly (p<0.001) increased to reached its peak in September (340.38 ng/dl) and October (491.5 ng/dl), respectively, later on testosterone quick dropped in November (Table 1 and Figure 3).

Plasma parameters

Some inorganic parameters of semen plasma were detected. These parameters of five males of Arabian Gazelles *G. subgutrosa marica* are illustrated in Table 2. The concentrations of sodium, potassium, chloride elements and fructose compound were measured during four months; February, May, August and November. Semen was collected every month from five males. Mean concentration of Sodium was 141±3.5 mM/L and the highest concentration was 147 mM/L in November. Mean concentration of calcium was 7.2±0.15 mM/L and the highest concentration was 7.2±0.15 mM/L in November. Mean concentration of chloride was 141±3.5 mM/L and the highest concentration was 129 mM/L in November. Mean concentration of fructose was 652.75±29 mg/dL and the highest concentration was 680 mg/dL in November. No significant changes in Semen plasma parameters were observed (Table 2).

Semen

Table 1 shows hormones (mean±SD) in males of Arabian

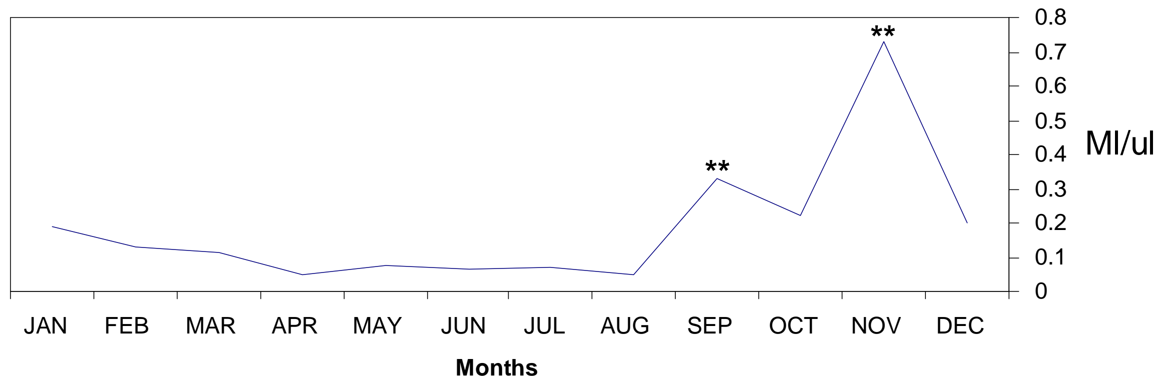

Figure 2. LH concentration during a year.

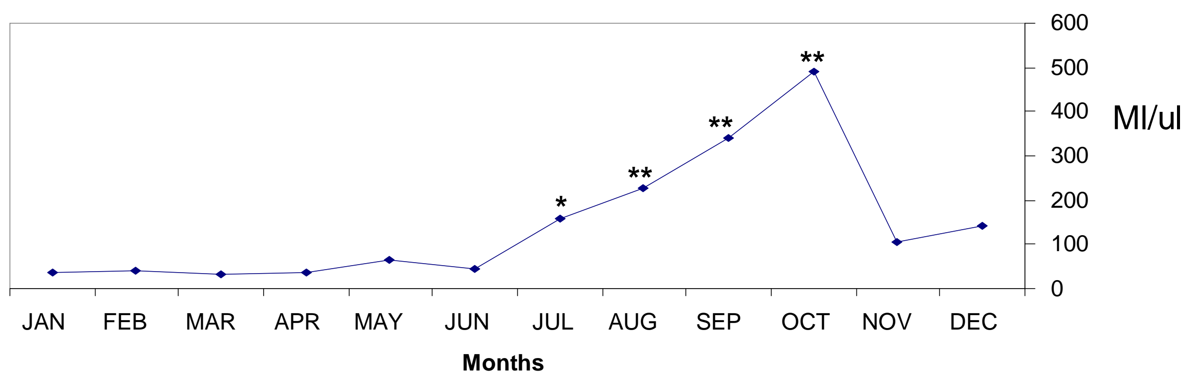

Figure 3. Testosterone concentration during a year.

Table 2. Semen plasma parameters (Mean±SD) in males of Arabian Gazelles (*Gazalla subgutrosa marica*) during the year (sample volume 0.5 ml).

Month	Na (mM/L)	K (mM/L)	Cl (mM/L)	Fructose (mg/dL)
Feb	610	122	7	140
May	655	124	7.3	142
Aug	666	121	7	135
Nov	680	129	7.5	147
Over Mean	652.75±29	124±2.3	7.2±0.15	141±3.5

Gazelles (*G. subgutrosa marica*) through the year (sample volume 5 ml). Table 2 shows semen plasma parameters (mean±SD) in males of Arabian Gazelles (*G. subgutrosa marica*) during the year (sample volume 0.5 ml).

DISCUSSION

The Arabian Sand Gazelles *(G. subgutturosa marica)* is classified as vulnerable (VU) on the IUCN Red List (IUCN Red, 2009). Scientific references on the Arabian Sand Gazelles concern only the ethological aspects and reintroduction as well as veterinary issues and diseases (KKWRC, 2008). The limitation of this study was the scant number of valued subjects (Chemineau, 1986). The information on reproductive physiology is still lacking. A few reports concerned these issues (Al-Eissa and Alhomida, 1997; Al-Eissa ,2007a; Al-Eissa et al., 2007b). Some studies described the reproductive season of Arabian Sand Gazelles and compared its reproductive cycles to Persian gazelle (*G. subgutturosa subgutturosa*) (Sempere, 2001). In the present study, concentration of FSH, LH and T slightly increased pre-sexual period. As

indicated in result, FSH hormones increased rapidly in October during mating season as shown in Table 1, and this revealed the important role of sex hormones in sexual period and also, its role in spermatogenesis. This data go in line with previous study on *Pudu puda* that showed increment of FSH during sexual period (George et al., 1996).

In contrast, LH hormone increased one month before FSH; this means that LH activated testosterone to enhance sperm premature. Again, this result agrees with that of George et al. (1996) on (*pudu puda).* Basically, increasing of Testosterone during three months of sexual period reflected on its fundamental functions that related to formation and preserve of spermatogenesis. This result supported (Gundogan, 2005) study that conducted on the sheep. There are evidences that a season influenced on both semen production and characteristics in some antelope breeds (Asher et al., 1993). In other antelope like goitred gazelles (*G. subgutturosa subgutturosa)* living in an arid environment conditions, intense sexual activity was observed during the breeding season (November to December) (Mambetdjumaev, 1970; Gorelov, 1972; Djevnerov, 1984; Blank, 1985).

Previous study reported morphometric, plasma testosterone, LH concentrations and seminal parameters in European fallow deer (Dama dama dama) and other genotypes. These results exhibited significant displacement between genotypes among year and revealed markedly seasonal patterns of secretion of testosterone and LH, with hybrid males exhibiting an apparent earlier onset of high-amplitude testosterone in February compared to those occurring in April for European males. Taken together, the data indicate strongly that the Mesopotamian influence is evident in the earlier attainment of sexual development and fertility in late summer and autumn, and earlier onset of sexual quiescence in spring (Asher et al, 1996).

The obtained data illustrated that the concentration of some inorganic semen compounds are non-significant increased during sexual period compared with other months. This result agrees with other studies that revealed the role of semen plasma to protect and enhance motility and activity of sperms (Gundogan, 2005) and protect sperms from cold shock (Barrios et al., 2000). However, it is very important to protect endangers species by all means of reproductive biotechnologies and understand the reproductive physiology of these animals, in order to increase the number of these threaten animals. In addition, extend the captive population and establish the more cryopreservation of genetic resources banks. Al-Eissa et al. (2007a) reported a breeding season in late summer and autumn with high quantity of semen volume and progressive spermatozoa. In gazelles males the breeding season in temperate areas seems to be different from the mountain gazelle. A possible hypothesis could be related to the Mountain gazelle different sensitivity to photoperiod or other influences on melatonin release (Al-Eissa, 2007a). Semen characteristics of mountain gazelle were highly influenced by season. The acceptable semen production to try cryopreservation programs was produced in October to December, but more investigations are needed in order to identify the exact breeding period. Frozen semen could be utilized in artificial insemination, for genetic studies, to increase genotypic variability and resolve the problem of rupicaprine species extinction (Al-Eissa, 2007a).

Conclusion

In conclusion, sexual hormones were strongly influenced by season. The results from this study suggest more investigation on the reproductive physiological research in order to detect the suitable period for semen collection of Arabian Sand Gazelles *(G. subgutturosa marica)* in Saudi Arabia. Also, to detect the suitable period for breeding in order to protect this animals and establish Cryopreservation of genetic resources banks in in Saudi Arabia for manipulation and long-term preservation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Short Communication

Selective isolation and screening of fungi with herbicidal potential and evaluation of herbicidal activity against *Vernonia* species

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In irrigated agriculture, weed control through chemical herbicides, creates various environmental and health hazards which lead to the search for an alternate method of weed management, which is an eco-friendly and effective means. Biological control of weeds using microorganism is now extensively studied to control various economic important weeds. In the present study, herbicidal activity of crude metabolites extracted from the fungal strains isolated from the soil was evaluated against *Vernonia* species. A total of 125 fungal soil isolates belonging to four fungal species such as *Alternaria alternata, Paecilomyces farinosus, Penicillium expansum,* and *Fusarium oxysporum* were isolated. The respective fungal isolates were cultivated in modified Fries media under standard condition. The mycelia and the filtrate were extracted with ethyl acetate and the concentrated extract was evaluated for the herbicidal activity adopting leaf necrosis assay. Among the different isolates, extract prepared from *A. alternata* and *P. farinosus* showed maximum herbicidal activity. The present study would suggest the possible use of the fungal extract as a bio-herbicidal agent after the effective formulation and mass production through standard conditions.

Key words: Herbicidal activity, fungi, extracts, *Vernonia species,* formulation.

INTRODUCTION

Weeds are an ever present and an increasingly significant constraint to agriculture production worldwide. The overall impact of weeds on crop production can be crudely calculated, with average loss varying from 10 to 20% (Charudattan and Rao, 1981). Current production levels can only be maintained through the regular and wholesale application of pesticides, particularly of chemical herbicides which account for almost 50% of agrochemical market (Woodburn, 1995). Chemical control has been internationally accepted but usage is restricted for the fact that effective weed herbicides can often affect many other plants, not to mention current public concern regarding environmental problems associated with chemical pesticide usage in general (Defago et al., 2001). Biocontrol agents (BCAs) have been recognized not only as a replacement for chemical pesticides, but also as a viable part of well designed, integrated weed management systems, utilizing living

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organisms or plant pathogens to control or reduce the population of undesirable weed species. Biological weed control is an approach utilizing living organism to control or reduce the population of an undesirable weed species (Watson, 1991). A bio-herbicide is a herbicide that is based on a living organism such as fungi, bacteria or protozoa. Bio-herbicides are employed to control weed species with a native pathogen applying them in massive doses in area infected with target weed flora. Bio-herbicides offer many advantages like high degree of specificity of target weed, no effect on non-target and beneficial plants or man; absence of weed resistance, development and absence of residue build-up in the environment (Karthick and Aruna, 2010).

Bio-herbicides include phytopathogenic microorganisms or microbial compounds useful for weed control. Collectively these organisms and/or their natural products are called bio-herbicides. Microorganisms produce secondary metabolites, which act on plants and other organisms which sometimes cause inhibition of growth, diseases and even death (Abbas et al., 2002). The initiative for using pathogens, phytotoxins from pathogens, and other microorganisms as biological weed-control agents began about three decades ago. Since then, numerous microbes have been screened for phytotoxic potential, and several dozen evaluated as bio-herbicides as reported by various researchers and summarized (Defago et al., 2001). Due to the interest in this area, many other weed pathogens and phytotoxins (from athogenic and nonpathogenic microorganisms) will be discovered that possess bio-herbicidal activity (Muller et al., 2009). Most bio-herbicides have been targeted toward agronomic weeds, but these agents may also be useful to control weeds in non-agronomic areas (recreational areas, forests, rights-of-way, lawns, gardens, etc.) where synthetic herbicides are not registered, or where their use is cost-prohibitive (Hussain et al., 1999). In the present study, herbicidal activity of metabolites extracted from fungi was evaluated against *Vernonia* species

MATERIALS AND METHODS

Isolation of fungi

The fungal isolates were isolated from local soil sample adopting soil dilution method (Clark, 1965). 25 g of soil sample was diluted in 225 ml of sterile distilled water. From this suspension, 10 ml was added to 990 ml of water and from this 1 ml was spread in Petri dishes in triplicates containing sabouraud dextrose agar (SDA) supplemented with chloramphenicol (100 mg/ml). The fungi were identified by colony morphology and microscopic staining with lactophenol cotton blue. The isolated fungi were maintained on sabouraud dextrose agar slant as monosporic culture.

Herbicidal activity

The herbicidal activity was done adapting detached leaf necrosis assay. The *Vernonia* plants were collected from university playground. Fresh leaves were collected in sterile polythene bags, kept in ice box, and brought to the laboratory immediately. Initially leaf necrosis assay was carried with all the isolated fungal conidia. The expanded leaves were surface sterilised with ethanol and washed with sterile distilled water to remove ethanol from surface. The leaves were inoculated with 10^8 spores/ml of isolated fungal conidia by wounding them with sterile needle on the surface of leaf and transferred to Petri plate containing moistened cotton ball and filter paper. Later plates were incubated at 25°C for one week. The fungal isolates that showed necrotic lesions on detached leaves was selected for further study. Among the 125 isolates, four fungal isolates: *A. alternata SKA08 strain, P. farinosus SKP01 strain, P. expansum SKPC01* and *F. oxysporum SKF01 strain* fungal conidia recorded herbicidal activity by the formation of necrotic lesions.

Crude extraction of herbicidal metabolites

The fungal extracts were prepared from mycelia and filtrate collected from modified Fries media. 500 ml of modified Fries media (sucrose 10 g; casein hydrolysates 2 g; sodium nitrate 1.5 g; dipotassium hydrogen orthophosphate 1 g; potassium chloride 0.5g; magnesium sulphate 0.5 g; ferrous sulphate 0.01 g; distilled water 1l; pH 6.8) was prepared and sterilised by autoclaving. 0.1 ml of spore suspension derived from 10 days old slant culture of *A. alternata SKA08 strain, P. farinosus SKP01, P. expansum SKPC01 strain, F. oxysporum SKF01* strain was inoculated and the inoculated flasks were kept at 28°C on a rotatory shaker at 150 rpm for 21 days. The broth was filtered through three layers of cheese cloth, the mycelia and filtrate collected was extracted with double the volume of ethyl acetate. The concentrated extracts was dissolved in DMSO and used for herbicidal activity. Leaf necrosis assay was done with aliquots of 10, 20 and 30 µg/ml of the concentrated extract as described earlier. The inoculated leaves with respective extracts were observed for development of necrotic lesions.

Formulation of herbicidal metabolites

The extract which showed maximum herbicidal activity was formulated in minimal media as liquid formulation. The minimal media (solution A: sodium phosphate pentahydrate 1.28 g; potassium phosphate (dibasic) 0.3 g; sodium chloride 0.05 g; ammonium chloride 0.1 g; distilled water 100 ml; solution B: magnesium sulphate 1.232 g/5 ml; solution C: calcium chloride 0.555 g/5 ml; solution D: glucose 2 g/10 ml; add 0.2 ml of solution B + 10 µl of solution C and 10 ml of solution D to solution A) was prepared and transferred to screw caps vials, sterilized by autoclaving. 1 ml of *A. alternata* mycelia and filtrate extract was added. One set of vials were kept for autoclaving and other set without autoclaving. Similarly one set of vials kept at 4°C for 48 h other kept at room temperature. Herbicidal activity was performed with all these formulation as described earlier.

RESULTS AND DISCUSSION

Fungi have a worldwide distribution, and grow in a wide range of habitats, including deserts, hypersaline environments, the deep sea, on rocks, and in extremely low and high temperatures. Fungi are the major group of organisms known to produce a wide range of metabo-lites, which shows several physiological changes in plant species known as phytotoxins (Yoshida and Hiradate, 1999; Abbasi et al., 2010). Such activity is called phyto-

Table 1. Diameter (cm) of leaf necrotic lesions caused by fungal extracts and re-extraction from necrotic lesions.

Name of fungi	Diameter of leaf necrotic lesions (cm)				
	Concentration (µg/ml)	Extraction		Reextraction	
		ME	FE	ME	FE
	10	0.8	0.4	0.4	0.9
Alternaria alternata	20	1.0	1.0	0.9	1.0
	30	1.8*	1.5	1.5	1.1
Spores		0.9			
	10	1.0	0.5	0.6	0.5
Fusarium oxysporum	20	1.2	0.9	0.8	0.6
	30	1.5	0.8	0.9	1.2
Spores		0.7			
	10	0.5	0.0	0.0	1.0
Penicillium expansum	20	0.7	0.0	0.0	1.2
	30	1.7*	0.0	0.0	1.5
Spores		0.6			
	10	1.0	0.2	0.4	0.7
Paecilomyces farinosus	20	1.5	0.6	0.7	0.8
	30	1.8*	1.2	1.0	1.0
Spores		0.9			

toxic activity. The metabolites of many fungi may have adverse or stimulatory effects on plants such as suppression of seed germination, mal formation and retardation of seedling growth reported that some fungal pathogens produce phytotoxins that effect seed germination and seedling growth and can be used to control various weeds (Hyang, 2008). In the present study, herbicidal activity of metabolites extracted from the fungal strain was evaluated against *Vernonia*. A total of 125 fungal strains were isolated from the soil. Among 125 isolates, herbicidal activity was recorded in *A. alternata SKA08* strain*, Paecilomyces farinosus SKP01, P. expansum SKPC01* strain and *F. oxysporum SKF01* strain spore treatment (Table 1). 0.9 cm of necrotic lesion was observed in *A. alternata* and *P. farinosus* followed by 0.7 cm in *F. oxysporum* and 0.6 cm in *P. expansum.* The extracts were prepared from all these 4 strains for the herbicidal activity. The mycelial and filtrate extract of all these strains showed necrotic lesions and concentration dependent variation on the development of necrotic lesions was observed. Maximum herbicidal activity was observed in *A. alternata* and *P. farinosus* mycelial extracts. The diameter of the necrotic lesion was found to be 1.8 cm at 30 µg/ml concentration. 0.8, 1.0 and 1.0, 1.5 cm of necrotic lesions was observed at 10 and 20 µg/ml mycelial extracts of *A. alternata* and *P. farinosus*. *F. oxysporum* also showed the herbicidal activity. The diameter of the necrotic lession was 1.0, 1.2 and 1.5 cm at 10, 20 and 30 µg/ml. *P. expansum* recorded necrotic lesions at the diameter of 0.5, 0.7 and 1.7 at respective concentrations. After re-extraction, all the tested strains mycelia and filtrate retained herbicidal activity. The diameter of the necrotic lesions was 0.9, 1.0, 1.1 in *A. alternata,* 0.5, 0.6. 1.2 in *F. oxysporum,* 1.0, 1.2, 1.5 in *P. expansum,* 0.7, 0.8, 1.0 cm in *P. farinosus* at respective concentration. The herbicidal activity of the extracts after reextraction from the necrotic lesions clearly reveals the virulence of the extracts. The filtrate extract also showed herbicidal activity. The filtrate extract of *A. alternata SKA08 strain* showed necrotic lesion at the diameter of 0.4, 0.9 and 1.5 cm at respective concentration. 0.4, 0.7 and 1.0 cm of necrotic lesion was observed in *P. farinosus SKP01 strain.* The diameter of the necrotic lesion in *F. oxysporum* extract was 0.6, 0.8, and 0.9 cm at respective concentration. But herbicidal activity was not recorded in *P. expansum* extracts at all concentrations. Similar effect was recorded in necrotic lesions after reextraction. No significant difference in the development of necrotic lesions was observed in re extraction treatment.

The liquid formulation of mycelia and filtrate extract of *A. alternata* in minimal media show distinct herbicidal activity. Autoclaved filtrate extract in minimal media showed maximum necrotic lesions of 1 cm diameter and mycelial autoclaved extract showed necrotic lesion of 0.6 cm diameter, but unsterilized refrigerated mycelial extract

caused maximum necrotic lesions. The diameter of necrotic lesion was 1.1 cm. Further study will be helpful to identify the mimicking of the pathogenic symptoms produced by fungal extracts of *A. alternata SKA08* strain on *Vernonia* species suggests a herbicidal role for the fungal extract in the necrotic lesions on the leaves of *Vernonia* species. Characterization of herbicidal compound in this fungal extract, mass production, formulation and herbicidal activity on other weeds (*in vitro* and field trial) will be carried out in future study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Partial purification and some physicochemical properties of *Aspergillus flavus* α-amylase isolated from decomposing cassava peels

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α-Amylase is one of the most important enzymes of great significance due to its wide area of potential application in food, fermentation, textile, paper, detergent and pharmaceutical industries. This study aimed at production of α-amylase from an indigenous fungal source and also ascertaining the properties of the enzyme for maximal activity. The enzyme was isolated from decomposing cassava peels, fractionated at 70% ammonium sulphate and characterized. The fungal isolate was characterized as *Aspergillus flavus.* The crude enzyme extract had a specific activity of 2.40 Umg^{-1} which increased to 7.88 Umg^{-1} on fractionation with ammonium sulphate with a yield of 11.10% and purification fold of 3.28. The K_m and V_{max} values of 0.52±0.009 g/dL and 62.57±0.23 U/min were obtained, respectively, at 2% cassava starch substrate. The enzyme also demonstrated maximum activity at 70°C and pH 5.0. It thus produces α-amylase which is thermostable, a property which could be exploited for industrial purposes where hydrolysis of starch and other complex carbohydrates are required.

Key words: Cassava, α-amylase, starch hydrolysis, *Aspergillus species*, industrial application.

INTRODUCTION

Amylases constitute a class of industrial enzymes representing approximately 30% of the world enzyme production (Calik and Ozdamar, 2001; Hmidet et al., 2008). α-Amylases (endo-1,4-α-D-glucan-4-glucanohydrolase: EC.3.2.1.1) constitute the family of endo-amylases which randomly cleave the 1, 4- α-D-glycosidic bonds between adjacent glucose units in the linear amylose chain retaining α-anomeric configuration in the products (Ramachandran et al., 2004). Although, amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand (Pandey et al., 2000). Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from

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fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al., 2006; Prabakaran et al., 2009; Alariya et al., 2013). It has been reported that fungi have high secretion capacity and are effective hosts for the production of foreign proteins (Tsukagoshi et al., 2001) with many species of *Aspergillus* and *Rhizopus* used as a source of fungal α-amylase (Gupta et al., 2008; Kim et al., 2011; Irfan et al., 2012). This probably could be due to the ubiquitous nature and non-fastidious nutritional requirements of these organisms (Abe et al., 1988). Specifically, starch degrading enzymes like amylases have received a great deal of attention because of their technological significance and economic advantages which include less time and space required for production, ease of process modification and optimization, cost effectiveness and consistency (Burhan et al., 2003).

α-Amylases are ubiquitous hydrolytic enzymes which play significant roles in the utilization of polysaccharides (Reddy et al., 2003). The ubiquitous nature, ease of production and broad spectrum of applications make α-amylase an industrially important enzyme. The production of α-amylase is essential for conversion of starches (an important constituent of the human diet and a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and cassava) into oligosaccharides. This polysaccharide hydrolysis yields products such as glucose, maltose, maltotriose, and α-dextrins (Stryer, 1995; Sajitha et al., 2011) which are of immense industrial use. The enzyme has numerous applications in brewing and fermentation industries for the liquefaction of starch to fermentable sugars; textile industry for resizing of fabrics; food industry for preparation of sweet syrups, to increase diastase content of flour and improve digestibility of some of the animal feed ingredients; paper industry for sizing (Rao and Satyanarayana, 2003; Aiyer, 2005; Sajitha et al., 2011). Others include detergent, pharmaceutical and fine chemical industries (Ashwini et al., 2011). Amylase is also used in the bread-baking process, to increase its volume and keeps its softness longer (Ammar et al., 2002). In addition, they are used in sewage treatment for reducing the disposable solid content of sludge (Parmar et al., 2001).

Meanwhile fungi belonging to the genus *Aspergillus* have been commonly employed for the production of α-amylase as posited that soils around mills and cassava farms after harvesting and treatment of tubers represent media where natural amylolytic activities occur, therefore, amylolytic microorganisms are expected to be present in these areas. Hence, this study was aimed at production and characterization of indigenous α-amylase with highest amylolytic activity from the fungi isolated from decomposing cassava peels.

MATERIALS AND METHODS

Phenylmethane-sulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Co., USA while Dinitrosalicylic Acid (DNSA) was obtained from BDH Chemicals Ltd., Poole England. Potato dextrose agar (PDA) was purchased from Oxoid Ltd. (Basingstoke, England). Other chemicals used were of analytical grade.

Isolation of organism and culture conditions

The fungi were isolated from decomposing cassava peels in a local cassava milling industry in Ikenne, Ogun State and cultured on Potato Dextrose Agar (PDA) incubated at 37°C for 7 days.

Screening of fungal isolates for amylolytic activity

The fungal isolates were sub-cultured on 1% starch-agar, pH 6.5 and incubated at 37°C for 7 days. These isolates were screened for amylolytic activity detected as clear zones after flooding with iodine solution. The fungus with maximum zone of hydrolysis was isolated and saved on a slant to be reactivated prior to use.

Identification of the isolate

The isolated organism was identified based on its morphological characteristics. The pure isolate of the fungus was obtained by repeated sub-culturing and used as inoculum in enzyme secretion.

Enzyme secretion and extraction

Prior to inoculation, the isolated organism on the slant was reactivated by sub-culturing on 1% starch-PDA agar plates and incubated at 37°C for 72 h. Thereafter, the growing culture was used as inoculum in sterile starch broth containing cassava starch (2.0 g/L), KH_2PO_4 (1.4 g/L), NH_4NO_3 (10 g/L), KCl (0.5 g/L), $MgSO_4.7H_2O$ (0.1 g/L), $FeSO_4.7H_2O$ (0.01 g/L) in replicates, pH 6.5 and incubated on a shaker incubator at 150 rpm for 72 h at 37°C. Culture was harvested and cells separated by centrifugation at 8,000 X g for 20 min at 4°C using a high speed cold centrifuge according to Ramachandran et al. (2004). The cell-free supernatant (crude enzyme) was assayed for the α-amylase activity and protein concentration.

Enzyme assay

The amount of reducing sugar liberated by the enzyme was measured using the method of Miller (1959); a modification of Bernfeld method (1955). The reaction mixture consisted of 1.25 ml pre-gelatinised 1% (w/v) cassava starch, 0.25 ml, 0.1 M sodium acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by adding 1.0 ml of 3, 5-dinitrosalicylic acid reagent followed by heating in a boiling water bath for 10 min and cooling at room temperature. The concentration of reducing sugar released was measured at 540 nm using glucose as the standard. One unit of α-amylase was defined as the amount of enzyme that releases one milligram of glucose under the assay conditions and expressed as:

$$\text{Alpha-amylase activity (U)} = \frac{\text{mg of glucose released} \times \text{dilution fold}}{\text{Time of incubation} \times \text{mg of enzyme in reaction mixture}}$$

Protein concentration determination

The protein concentration of the enzyme was determined using

Table 1. Purification table of *Aspergillus flavus* α-amylase from decomposing cassava peels.

Step	Fraction	Volume (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
1	Crude extract	760.00	3120.00	7491.20	2.40	100.00	1.00
2	70% Ammonium sulphate fractionation	16.00	105.60	832.40	7.88	11.11	3.28
3	Dialysate	25.00	67.50	2447.55	36.26	32.67	15.64

Biuret method (Gornall et al, 1949) using bovine serum albumin (BSA) as standard. The protein concentration of each purification step was then extrapolated from the standard protein calibration curve.

Ammonium sulphate fractionation

The crude enzyme was brought to 70% ammonium sulphate saturation by adding slowly, followed by gentle stirring of the solid ammonium sulphate (472 g/L) in an ice cold environment. The stirring continued occasionally until all the salt had been dissolved. The mixture was then allowed to stand overnight at 4°C. The precipitated protein was recovered by centrifugation at 8,000 X *g* at 4°C for 20 min using a high speed cold centrifuge. The supernatant resulting from the 70% ammonium sulphate fractionation was discarded while the precipitate was kept and re-suspended in a minimal volume of 10% ammonium sulphate in 0.01 M Sodium acetate buffer. The enzyme activity and protein concentration were also determined.

Effects of pH

The optimum pH of the enzyme was determined by the measurement of activity of the partially purified enzyme at different pH values ranging from 4.0 to 9.0 using three buffer system comprising: 0.1 M sodium acetate buffer at pH 4.0 to 5.0, sodium phosphate buffer (pH 6.0 to 8.0), and Tris-HCl buffer (pH 9.0) while assay was carried out routinely.

Effect of temperature

Optimum temperature of the enzyme was determined by routine measurement of the activity of the enzyme by incubating at temperatures between 40 to 100°C for 10 min.

Determination of kinetic parameters

The kinetic constants K_m and V_{max} were determined using aqueous 2% cassava (pre-gelatinised) starch solution as substrate. The reaction mixture contained 1.25 ml of the substrate, 0.25 ml of appropriately diluted enzyme solution, 0.10 ml of 80 mM $CaCl_2$ and 0.25 ml of 0.10 M sodium acetate buffer. 0.40 ml of distilled water was added to the reaction mixture and incubated at 50°C for 10 min after which 1.0 ml of dinitrosalicylic acid (DNSA) was added and heated at 100°C for 10 min. On cooling, the absorbance was read at 540 nm.

RESULTS

Morphological identification of the Isolate

Four fungi were observed after culturing the sample and the one with the highest amylolytic activity, that is, the fungus having largest zone of hydrolysis on flooding with iodine was isolated and morphologically characterized. The colonies of the pure strain of the fungus showed pale-yellowish colouration; centrally rising with close textured velvety and regular margins. This fungus was identified as *A. flavus* and used for further studies.

Enzyme purification

The purification table of the *A. flavus* α-amylase is shown in Table 1. The total protein concentration and activity of the crude extract were 3120.0 and 7491.20 mg while on fractionation at 70% ammonium sulphate it gave 105.60 mg of total protein concentration and activity of 832.40 U. The result of varying substrate concentration with reaction velocity resulted in hyperbolic curve and the kinetic constants (K_m and V_{max}) were determined from the Line weaver-Burk's plot (Figure 1). The maximum velocity, V_{max} obtained was 62.57±0.23 U/min while the Michaelis-Menten constant, K_m was 0.52±0.009 g/dL. The optimal temperature of 70°C and pH 5.0 were obtained for the *A. flavus* α-amylase (Figures 2 and 3), respectively.

DISCUSSION

Fungi are known to utilize polysaccharide substrates through extracellular digestion catalyzed by secreted enzymes. This high capacity secretory system has driven the exploitation of filamentous fungi as cell factories for provision of enzymes used in a wide variety of applications (Archer, 2000). *A. flavus*, a filamentous fungus isolated from decomposing cassava peels was identified in this study, an indication that the organism was able to utilize starch, a naturally occurring glucose polymer in plants, as food. This genus has been reported to produce a large variety of extracellular enzymes with amylases being the most significant and of industrial importance (Hernández et al., 2006) which are known to produce considerable quantities of enzymes that are used extensively in the industry. Specifically, *A. flavus* had been reported to secrete α-amylase (Ali and Abdel-Moneim, 1989). Abou-Zeid (1997), isolated filamentous fungi from cereals and screened to test the alpha-amylase producing potential. The strain which showed

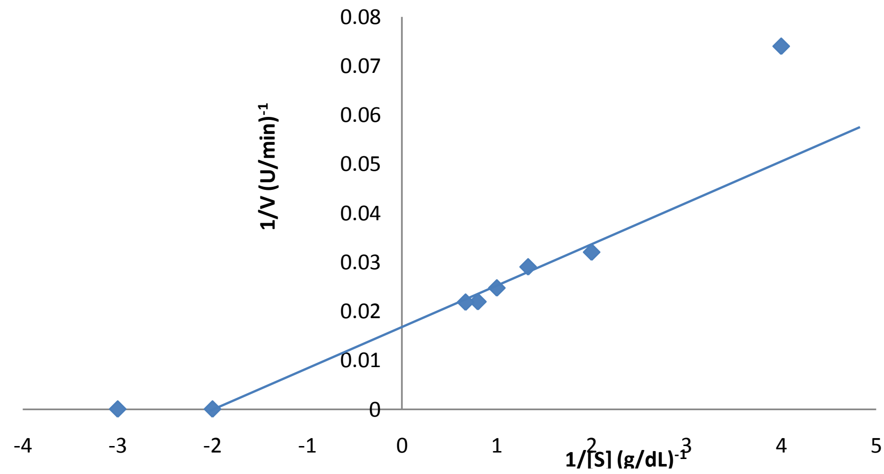

Figure 1. Lineweaver-Burk's plot of the kinetic parameters of the *Aspergillus flavus* α-amylase.

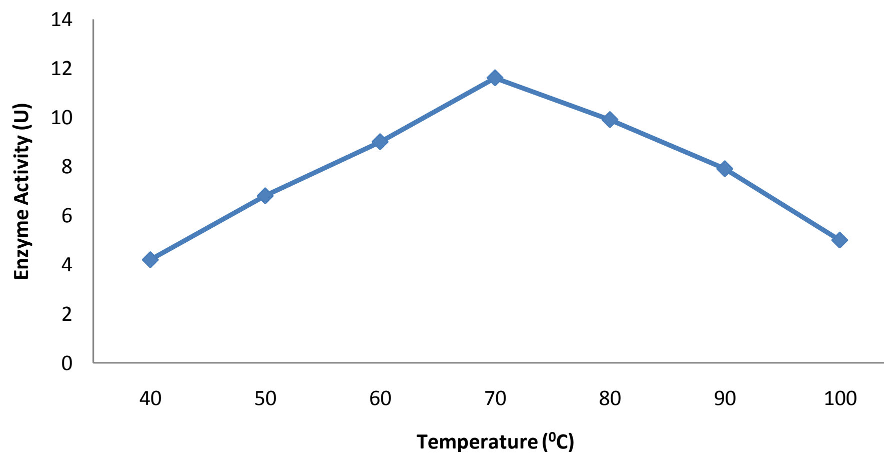

Figure 2. Temperature profile of the *Aspergillus flavus* α-amylase.

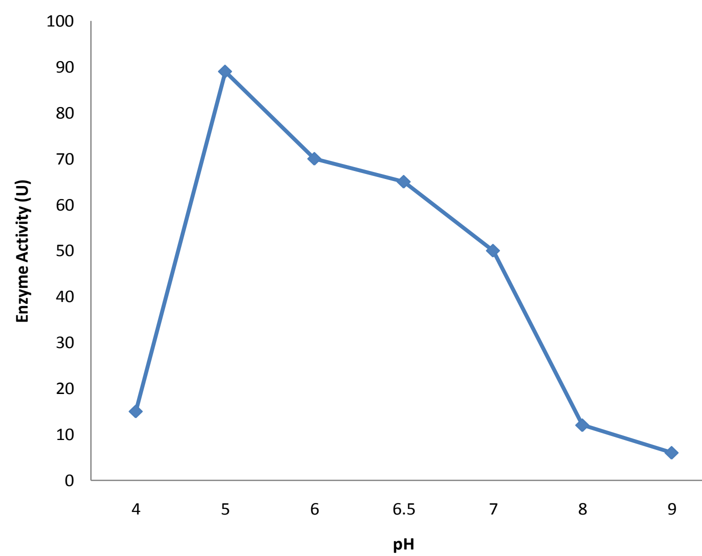

Figure 3. pH profile of the *Aspergillus flavus* α-amylase.

highest ability for α-amylase production was identified as *A. flavus*. Likewise, a species of the genus *Aspergillus oryzae* has received increased attention as being a favourable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, for example α-amylase (Jin et al., 1998). Different investigators have reported differing specific activity values of the enzyme isolated from various sources: *A. oryzae* from mangrove associated fungi (2.29±0.03 µmol/min/mg; Joel and Bhimba, 2012) and *A. oryzae* from waste water (8.77 IU/mg; Shah et al., 2014). The specific activity of 7.88 U/mg obtained at 70% ammonium sulphate fractionation in this work is not all that different from value reported (Shah et al., 2014), though on dialysis it increased to 36.26 U/mg which reflected progress of the purification procedure. It is pertinent to mention that *A. flavus* α-amylase in this study obeyed Michaelis-Menten kinetics as evident in the Line weaver-Burk's plot (Figure 1), a pattern also corroborated by Shah et al. (2014) who also reported the K_m value of 1.4 mg/ml, a value lower than 0.52±0.009 g/dL obtained in study though substrate concentration also differs. The K_m value was known as the criterion for the affinity of enzymes to substrates, and lower value of K_m represents higher affinity between enzymes and substrates (Shuler and Kargi, 2002). The reverse was the case for V_{max} where it was 62.57±0.23 U/min in this study and 46.56 IU/mL by same researchers.

The optimum temperature of 70°C obtained in this study for the *A. flavus* α-amylase is higher than the optimum temperature of 30°C reported for the same enzyme sourced from cereal (Abou-Zeid, 1997); 40°C for *Rhizobium s*train (Oliveira et al., 2010) and temperature range between 50 to 55°C for the thermophilic fungal cultures such as *Talaromyces emersonii*, *Thermomonospora fusca* and *Thermomyces lanuginosus* (Jensen and Olsen, 1992; Bunni et al., 1989) and 50°C for *Aspergillus niger* α-amylase isolated from potato (Siddique et al., 2014). Thus, the strain of the fungus isolated in this study is one of very few with this thermophilic property producing the thermostable α-amylase. pH is another factor that determines the growth and morphology of the microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi required slightly acidic pH for optimum growth (Sivaramakrishnan et al., 2006). pH is known to affect the synthesis and secretion of alpha-amylase just like its stability (Fogarty, 1983). Fungi of *Aspergillus sp*. such as *A. oryzae, Aspergillus ficuum* and *A. niger* were found to give significant yields of α-amylase at pH 5.0 to 6.0 in submerged fermentations (Moller et al., 2004); Knox et al., 2004). Therefore, the pH optimum 5.0 obtained in this work compares well with the works of Ali and Abdel-Moneim (1989); Sudo et al. (1994) and Odibo and Ulbrich-Hofmann (2001) who reported optimum pH of 5.0 for alpha-amylase from *A. flavus var. columnaris, Aspergillus kawachi* IFO 4308 and *T. lanuginosus* respectively which indicated that the organism prefer acidic condition for better enzyme production.

Conclusion

The organism therefore is regarded as a viable local strain since it secreted α-amylase with thermostability property at 70°C. This could therefore be exploited for use in liquefaction of starch and or other industrial purposes where hydrolysis of starch and other complex carbohydrates are required.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Short Communication

An active form of calcium and calmodulin dependant protein kinase (ccamk) of *Medicago truncatula*

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The *DMI3* gene of the model legume *Medicago truncatula* encodes a calcium and calmodulin dependent protein kinase (CCaMK) involved in the signalling pathways leading to the establishment of both mycorrhizal and rhizobial root symbiosis. The removal of the auto-inhibitory domain that negatively regulates the kinase activity in *M. truncatula* results in a constitutively-active form, inducing symbiotic responses in the absence of bacterial signals. In this study, we verified the functionality of a *DMI3* variant and its ability to induce spontaneous nodules in *M. truncatula dmi3* mutant. Our results based on enzymatic radio activity assay using [γ-^{33}P] ATP, suggests that the DMI3-311 variant is active and its corresponding gene (*DMI3-311*) when introduced in the *dmi3* mutant, by *Agrobacterium rhizogenes* transformation, induced in the formation of a few spontaneous nodules.

Key words: *Medicago truncatula*, CCaMK; *DMI3* variant; spontaneous nodules.

INTRODUCTION

Legumes can form a nitrogen fixing symbiosis with soil bacteria called rhizobia (the RL symbiosis). They can also like most plants, form symbiotic associations with arbuscular mycorrhizal (AM) fungi, which facilitate plants' phosphate nutrition. In both interactions, the symbionts are hosted inside the plant root. Nitrogen-fixing rhizobia are housed in intracellular symbiotic structures within nodules, while AM fungi form intracellular symbiotic structures called arbuscules, within cortical root cells and can also develop external mycelium which extends from around the root. Molecular genetics studies performed on the model legumes *Medicago truncatula* and *Lotus japonicas* have shown that the establishment of the nodulation and the mycorrhization processes share a common signaling pathway, required for the initiation of endosymbiotic programs in host plants. In *M. truncatula*, three genes called the *DMI* genes (which does not make infection) are involved in this pathway (Catoira et al., 2000). The common signaling pathway diverges after *DMI3* which represents the last known gene common to both symbiosis. *DMI3* encodes calcium and calmodulin dependent protein kinase (CCaMK), located in the nucleus, and is supposed to perceive and transduce calcium signals generated upon perception of the symbiotic signals. Therefore, it has been hypothesized that *DMI3*, depending on its activation determined by the calcium signature, could phosphorylate substrates involved either in nodulation or mycorrhization (Levy et al., 2004).

CCaMKs has been studied in other plants and its activity depends on an auto-inhibitory domain that negatively

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regulates the kinase activity. Removal of this domain or point mutations at the auto-phosphorylation site respectively, in *M. truncatula* and *L. japonicus*, leads to a constitutively-active form of CCaMK that can induce spontaneous symbiotic responses and nodulation in the absence of Nod factors (Gleason et al., 2006; Tirichine et al., 2006). Otherwise, a protein interacting with *DMI3*, named IPD3 (interacting protein of *DMI3*) in *M. truncatula* and CYCLOPS in *L. japonicus*, has been identified (Messinese et al., 2007; Yano et al., 2008). CYCLOPS, which is phosphorylated *in vitro* by the CCaMK seems to be important for the infection process in both symbioses, but is dispensable for nodule organogenesis, suggesting that CCaMK has different molecular targets during the nodulation process. Recently, Rival et al. (2012, 2013) showed that both rhizobial and mycorrhizal symbiosis are controlled by *DMI3* in a cell autonomous way. Nodule organogenesis was not observed when *DMI3* expression was restricted either to the epidermis or to the cortex, but was restored when *DMI3* was expressed in both tissues. Moreover, it has been shown that transcription factor NIN downstream DMI3, negatively regulates infection but positively regulates nodule organogenesis during the course of the symbiosis (Yoro et al., 2014). Through all these observations, the infection process needs the full activation of CCaMK *via* the calcium spiking generated by the common signalling pathway and possibly an additional calcium signal. In this context, the use of a constitutively-active form of *DMI3* could be a valuable tool to search for downstream targets which could potentially include further components of the common symbiotic signaling pathway or components specific to Nod or Myc signalling. From previous work, *DMI3* variant (DMI3-311) in terms of production was described as a tool for identifying substrates potentially involved in nodulation or mycorrhization (Kassouar and Baba Hamed, 2011). In this study, we verified the functionality of this variant and its ability to induce spontaneous nodules in the *M. truncatula dmi3* mutant.

MATERIALS AND METHODS

Enzymatic radio activity assay

The catalytic activity of the recombinant DMI3-311 was examined by a test of auto-phosphorylation of the protein. Indeed, a volume of 40 µL purified fraction was added to a reaction buffer (10 mM $MgCl_2$, 50 µM [γ-^{33}P] ATP, 1mM DTT and 10 mM $MnCl_2$), the mixture was then incubated at 25°C for 1 h. After centrifugation, the labeled proteins were separated by SDS-PAGE gel electrophoresis (10% acrylamide). The labeling of phosphorylated proteins was revealed on X-Ray film (Amersham) after 4 to 5 days of exposure at -80°C.

M. truncatula transformation by *Agrobacterium rhizogenes*

Transformation of *dmi3* mutant by *Agrobacterium rhizogenes* using pCambia2202 binary vector with *DMI3 1-311* construction (the tagged form: StrepTagII and no tagged form were used separately) under control of its own promoter (*pDMI3-DMI3 1-311*) (Godfroy et al., 2008), began with (TRV25 allele) *dmi3* mutant germination. After the step of scarifying by H_2SO_4 (95%), surface-sterilised seeds were sown on agar plates and placed for 3 days in the dark at 4°C then left overnight at 25°C to germinate. After approximately 30 h germination, when seedlings had a radicle length of approximately 10 mm, the radicle was sectioned approximately 3 mm from the root tip with a sterile scalpel. Sectioned radicles were inoculated by coating the freshly cut surface with *A. rhizogenes* grown on TY solid medium (Boisson-Dernier et al., 2001). Thereafter, the inoculated sectioned seedlings were placed on slanted agar (Laboratoire Industriel de Biologie, Avignon, France) containing a modified Fahraeus medium supplemented with kanamycin (1mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 50 µM FeEDTA, 0.5 mM NH_4NO_3, supplemented with 0.1 mg of $MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3, and Na_2MoO_4 per liter) in square Petri dishes (12 × 12 cm). After several incisions in the Parafilm seal allowing gas exchange, the Petri dishes were placed vertically in a growth chamber at 20°C for 1 week (16-h photoperiod and a light intensity of 70 µE/s/m^2) to optimize the transformation frequency, and then transferred to a 25°C growth chamber (identical light conditions) for 2 weeks.

For nodulation assays, transformed plants were transferred separately in two different environments: nto growth pouches, with 7 ml of Fahraeus liquid medium (with nitrates) (3 to 4 plants/pouch), and incubated at 25°C for 3 to 4 weeks; and to sepiolite (Agrauxine, Quimper)/sand (2:1 volume mix) pots and grown at 25°C with 18 h light/6 hour dark cycles (Catoira et al., 2000).

Microscopy methods

The presence of spontaneous nodules was first observed by optical microscope (immersion x100) (Zeiss Axiophot, Carl Zeiss, Germany). Transformed roots were cleared with 3% sodium hypochlorite for 15 min and observed with a Leica MZFLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany).

RESULTS AND DISCUSSION

In order to identify targets DMI3, a biochemical study based on obtaining a truncated form of DMI3 displaying constitutive activity was chosen to overcome stimulation by Nod factors and providing DMI3 homogeneous samples that would be permanently in its interaction with potential targets. For this we followed Gleason et al. (2006) who showed that expression of a truncated form of DMI3 in the roots of *M. truncatula* induces symbiotic responses in the absence of symbiont. These researchers present the construction of a set of partially-deleted mutants to DMI3, 1-326 and 1-311, corresponding to the kinase domain without two regulatory domains (visinin domain and central domain). *In vitro* phosphorylation tests on artificial substrate showed constitutive activity, independent calcium, 1-326 and 1-311 forms of DMI3, corresponding to about 30 and 40%, respectively of the activity of the whole protein in the presence of calcium and calmodulin. From previous work done by authors, a truncated form of DMI3 was produced, the variant DMI3-311. Here, the catalytic activity of this variant was first verified, *in vitro*, by autophosphorylation assay of the protein.

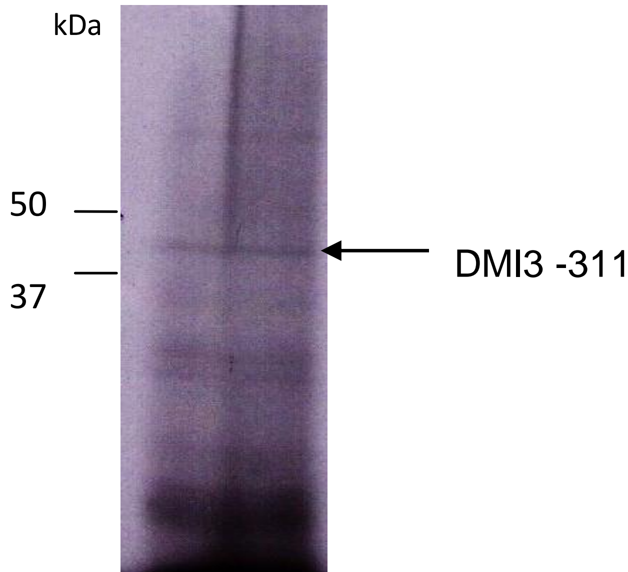

Figure 1. Enzymatic radio activity assay. Radioenzymatic labeling of the protein with [γ-^{33}P] ATP, reveals the presence of phosphorylation at the expected molecular weight about 39 to 42 kDa confirming the functionality of the DMI3-311 protein.

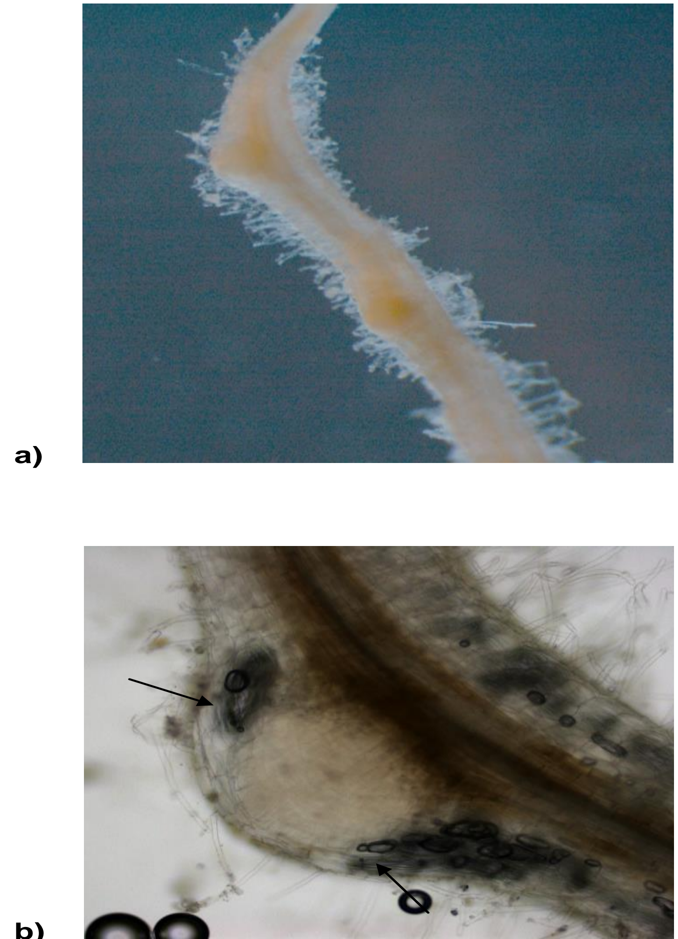

Figure 2. Spontaneous nodules in the *M. truncatula dmi3* mutant grown on sepiolite. After transformation of *dmi3* mutant by *Agrobacterium rhizogenes* using the binary vector *pCambia*2202 with *DMI3 1-311* construction under control of its own promoter (*pDMI3-DMI3 1-311*), complement plants were then transferred to sepiolite (Agrauxine, Quimper)/sand (2:1 volume mix) pots and grown at 25°C with 18 h light/6 h dark cycles. Spontaneous nodules observation in the *dmi3* mutant transformed with *DMI3-311* no tagged construction: **a)** by optical microscopy (immersion x100) (Zeiss Axiophot, Carl Zeiss, Germany), allowing us to visualize transparent rounded nodules. **b)** after roots clarification (3% sodium chloride for 15 min) and microscopic observation by a Leica MZFLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany). The presence of peripheral vascular bundles (arrow) is characteristic of nodule anatomy in *M. truncatula*.

Catalytic activity of the DMI3-311 protein

The presence of auto-phosphorylation was detected using a radioenzymatic labeling of the protein with [γ-^{33}P] ATP. It is important to know that at the end of the N-terminal kinase domain is found the binding domain to ATP and at its C-terminal the Thr267, auto-phosphorylation site of the protein (Sathyanarayanan et al., 2000; Sathyanarayanan and Poovaiah, 2002). Figure 1 reveals the presence of phosphorylation at the expected molecular weight about 39 to 42 kDa confirming that the DMI3-311 recombinant protein is active.

Spontaneous nodulation

We tested, *in planta*, the ability of the variant DMI3-311 to induce spontaneous nodules in absence of bacterial symbiont and Nod factor. For this, we performed tests of complementation of *dmi3* mutant using the binary vector *pcambia*2202 with *DMI3 1-311* construction under control of its own promoter (*pDMI3-DMI3 1-311*) (Godfroy et al., 2008) (the tagged form: Strep TagII, and no tagged form were used separately) by *Agrobacterium rhizogenes* transformation. Composite plants were then transferred separately in two different environments, into growth pouches with Fahraeus liquid medium and to sepiolite pots. The transformation efficiency, in absence of any stimulation symbiotic (Nod factors or bacterial symbiont) results first, by the early nodulin *ENOD11*gene expression sion conferring a blue coloration to root epidermic tissue (data not shown) and the formation of structures with the anatomy of a nodule. Few spontaneous nodules were observed in plants complemented with the no tagged *DMI3 1-311* construction and grown in sepiolite (Figure 2). Microscopic observations revealed transparent rounded nodules (Figure 2a) characterized by the presence of peripheral vascular bundles characteristic of nodule anatomy in *M. truncatula* (Figure 2b). These results were confirmed after histological assay (data not

shown). However, no nodules were observed in plants complemented with the StrepTagII *DMI3 1-311* construction.

In light of these results, we can claim to be in possession of a constitutively active truncated form of the kinase DMI3, this molecular tool will allow us to achieve phosphorylation *in vitro*, first in extracts of cell nuclei suspensions of *M. truncatula* A17 (wild strain) and the mutant *dmi3* (as suspension cells are easy to grow) and in extracts of root, in order to identify substrates or DMI3 molecular partners involved in the process of nodulation or mycorrhiza. Preliminary *in vitro* phosphorylation studies, on cell suspensions nuclei extracts of *M. truncatula A17* (wild type) and *dmi3* mutant are encouraging but need to be confirmed.

Conclusion and recommendation

By decoding calcium signals, *DMI3*/CCaMK could play a central role in orientating the signalling pathway leading to nodulation or mycorrhization. The possibility to produce active DMI3-311 gives the opportunity to search for its substrates by performing *in vitro* phosphorylation assays using plant extracts.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

In vitro anthelmintic effects of crude aqueous extracts of *Tephrosia vogelii, Tephrosia villosa* and *Carica papaya* leaves and seeds

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The prevalence of anthelmintic resistance and the consumer demand for alternative farming systems that limit the use of chemical anthelmintics has made the search for alternative gastrointestinal nematode parasites control methods crucial. Traditional medicinal/herbal plants can offer an alternative to the reliance on chemical anthelmintic drugs. This study evaluates the efficacy of crude aqueous extracts of *Tephrosia vogelii* Hook., *Tephrosia villosa* Pers., and *Carica papaya* Linn. leaves and *Carica papaya* Linn. seeds against gastrointestinal nematodes using *in vitro* egg hatch and larval development inhibition assays. Rectal faecal samples from sheep were subjected to parasitological examination for faecal egg counts (FEC) using the McMaster counting technique. 100 g of dried and poultice aqueous leaf extract of *T. vogelii, T. villosa*, *C. papaya* leaves and seeds was blended into liquefaction in 200 ml of distilled water then boiled at 90-100°C for 1 h and cooled. Levamisol and distilled water were used as positive and negative control in the bioassay. Egg hatch assay revealed more than 95.8% reduction in egg hatch at concentration of 500 mg/ml for dried and poultice paste of *T. vogelii* leaves and *C. papaya* seeds. Larval development inhibition assay results showed that both dried and poultice paste of *T. vogelii* leaves and *C. papaya* seeds extract yielded more than 98% inhibition at a concentration of 500 mg/ml. Based on the LD_{50} dried extract of *C. papaya* seeds was most potent extracts for the inhibition of both egg hatching (49.94 mg/ml) and larval development (49.32 mg/ml). Both poultice and dried extract for all the plants showed significant and dose dependent egg and larval development inhibition. These findings indicate that the evaluated plants have potential anthelmintic effect and could provide viable alternatives for the control of gastrointestinal helminthes in ruminants.

Key words: Aqueous extracts, Anthelmintic activity, Medicinal plants, *Tephrosia vogelii*, *Tephrosia villosa*, *Carica papaya*.

INTRODUCTION

Helminthosis adversely affect ruminants productivity and welfare in both organic and conventional systems (Silva et al., 2011; Chartier and Paraud, 2012; da Silva et al., 2013). It is ranked the highest animal health constraint to

the poor especially in tropical and sub-tropical countries (Perry et al., 2002). There are a number of approaches used to control helminthes in livestock, including nutritional, immunological and biological interventions (Jackson and Miller, 2006). However, most farmers rely on chemical anthelmintic drugs. The cost and non-availability of synthetic anthelmintic for some farmers, emergence of drug resistance, environmental pollution and toxic chemical residues reported in foods derived from livestock are a major cause of concern for many consumers (Jackson and Coop, 2000; Kaplan, 2004; Saddiqi et al., 2010; Sutherland and Leathwick, 2011). Therefore naturally occurring plants with anthelmintic properties could offer alternatives that can overcome some of these problems.

Consumers demand more natural and higher quality foods (Casemiro and Trevizan, 2009). This is due to the growing demand for healthy foods for the people and awareness of impact of chemical residues on the environment. There is a worldwide debate about the development of sustainable food production systems, adapted to different farming conditions. Alternative concepts of agroecology and holistic agriculture, that advocate for the use of integrated management strategies, such as target selected treatment, herbal medicine, and the application of other parasite control alternatives, are undergoing resurgence because of their more sustainable appeal (Molento, 2009). Use of medicinal plants could offer possible alternatives that may be important for agroecological production systems, organic or biological - dynamical systems where the use of chemical drugs is limited (Peixoto et al., 2013).

Tephrosia vogelii Hook. is widely used across Africa as a fish poison, pesticide and for soil enrichment (Neuwinger, 2004; Mafongoya and Kuntashula, 2005; Sirrine et al., 2010; Kamanula et al., 2011). The methanolic leaf extracts have shown anthelminthic activity against *Nippostrongylus braziliensis* (Edeki, 1997). *Carica papaya.* Linn is popularly used as a dessert or processed into jam or wine, while the green fruits are cooked as vegetables (Samson, 1986; Nakasone and Paul, 1998). *C. papaya* is among the 13 plant species used by farmers as anthelmintics to combat worm infestation in livestock in Nigeria (Adedapo et al., 2002). Aqueous extracts of papaya seeds have shown anthelmintic activity against *Haemonchus contortus*, *Trichostrongylus* spp., *Strongyloides* spp. and *Ostertagia* spp. in Sheep (Ameen et al., 2010). Kermanshai et al., (2001) identified benzyl isothiocyanate has the predominant or sole anthelmintic agent in papaya seed extracts against *Caenorhabditis elegans*. Among these botanical species, *C. papaya* (pawpaw) may be preferred as an ethnoveterinary remedy in this part of the tropics because of its adaptability, agro-ecological considerations and availability (Mundy and Murdiati, 1991).

There is, however, no scientific evidence for the anthelmintic effects of *Tephrosia villosa* Pers. The plants were selected and evaluated based on the indigenous knowledge information about their use by farmers against helminthes. The plants are also distributed widely in Kenya and an assessment of their possible efficacies was considered to be of interest.

The study was conducted to evaluate the *in vitro* anthelmintic activity of aqueous extracts of *Tephrosia vogelii* Hook., *Tephrosia vellosa* Pers. and *Carica papaya* Linn. leaves and *Carica papaya* Linn. seeds to validate their use in ethnoveterinary medicine among some farmers in Kenya. These tests are based on the hypothesis that an anthelmintic activity observed *in vitro* would be indicative of a potential *in vivo* activity.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaves of *T. vogelii* and *C. papaya* were collected on May 2013 at the Kenya Agricultural Research Institute - National Research Laboratory (NARL) in Nairobi while fresh leave of *T. villosa* were collected from the Kenya Agricultural Research Institute station in Kiboko. *C. papaya* Linn. seeds were collected from ripe pawpaw fruits and washed with clean water to remove dirt. The plants were identified and authenticated in the Department of Botany at the National Museums of Kenya, Nairobi and voucher specimens of each species were deposited at the University of Nairobi herbarium.

The plant materials and seeds were divided into two samples for each plant species. The first samples were ground soon after collection to make a poultice paste of 100 g which was blended into liquefaction in 200 ml of distilled water then boiled at 90-100°C for 1 h and cooled. The second set of samples were dried in shade at ambient temperature for 14 days, ground and milled to powder by electrical blender.

100 g on the powder was also blended into liquefaction in 200 ml of distilled water boiled at 90-100°C for 1 h and then cooled. Both samples were then centrifuged at 1500 rpm for 5 min. The supernatant was filtered through sterile filter papers and stored at 4°C in dark tightly closed glass bottles until used. One millilitre of the filtrate contained 0.5 g (500 mg/ml) of the extract.

Preparation of serial dilutions of aqueous extracts

Serial dilutions of stock solution were performed to yield 10 ml each of 500, 250, 125 and 62.5 mg/ml concentrations of the extract.

Recovery and preparation of eggs

Fecal materials (pellets) were collected per rectum from sheep with natural acute/sub-acute parasitic gastroenteritis due to mixed nematode species. The samples were placed into labeled specimen

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bottles and transported to the Laboratory at the Department of Veterinary Pathology, Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Nairobi. All the samples collected were processed on the same day.

Fecal samples were examined for helminthes eggs using the modified McMaster technique described by Hansen and Perry (1994). Briefly, approximately 3 g of feces were placed in a beaker and 45 ml of floatation fluid (saturated sodium chloride solution) added. The feces were broken into pieces and mixed by stirring with a wooden spatula. The mixture was sieved using a tea strainer into another beaker and subsample taken from using Pasteur pipette while stirring. A McMaster counting chamber was filled with the subsample and the number of eggs counted under a microscope at X40 magnification.

Egg hatch assay

The *in vitro* egg hatch assay method described by Coles et al. (1992) was adopted. A suspension of 20 µl was distributed in three 96-flat-bottomed microtiter plates containing approximately 100 fresh eggs per well and mixed with the same volume of plant extract having different concentrations (500, 250, 125 and 62.5 mg/ml). Four other similar replicates of the plates were made to evaluate the effect of the plant extracts over a three day period. In the control plates levamisol and distilled water was added to the egg suspension. Levamisole was used only at one dose level of 3.125mg/ml as a reference drug. The eggs were incubated in this mixture for 48 h at 27°C and 70% relative humidity. After 48 h, a drop of Lugol iodine solution (Reidel de Hae) was added to stop the eggs from hatching. Hatched larvae (dead or alive) and unhatched eggs were then counted under dissecting microscope.

An inhibition percent (%) of egg hatching was calculated for each extract concentration using the following modified formula of Coles et al. (1992):

$$\text{Inhibition (\%)} = 100 \times (1-X_1)/X_2$$

Where, X_1 is the number of eggs hatched in test extracts, and X_2 is the respective number in distilled water control.

Larval development and viability assay

The procedure used was a modification of the technique described by Hubert and Kerbouef (1992). Aliquots of 150 µl of a suspension with about 100 eggs per well and 20 µl of filtrate obtained by faecal washing during egg recovering were distributed to wells of a 96-well flat-bottomed microtiter plates. This suspension was supplemented with 30 µl of the nutritive medium described by Hubert and Kerboeuf (1984) and comprised of Earle's balanced salt solution (Sigma) plus yeast extract (Sigma) in saline solution (1 g of yeast extract/90 ml of saline solution) at a ratio of 1:9 v/v. The plates were incubated at 27°C and 70% relative humidity. After 48 h, 200 µl of the plant extracts at same concentrations as mentioned above, levamisol and distilled water (control) were added to respective plates. There were four replicates for each extract concentration and control. The plates were further incubated for five days (total of seven days), further development was stopped by addition of one drop of Lugol`s iodine solution. All L1 and L3 larvae in each well were counted under a dissecting microscope. The percentage of development was calculated as the ratio: number of L3/total number of larvae. The percent mortality was calculated from an average of the four replicates.

Statistical analysis

The data from egg hatch assay/test and larval development assay/-test were transformed by probit analysis against the logarithm of extract concentration using SPSS for Windows version 16.0. The extract concentration required to inhibit 50% (LD_{50}) egg hatching and 50% (LC_{50}) larval development were calculated after correction for natural mortality by probit analysis. The comparisons of mean percentage of egg hatching and larval development inhibition at different concentrations with the control, was done by one way analysis of variance (ANOVA). All statistical analyses were performed by SPSS version 16.0 for windows. The post hoc statistical significance employed was the least square difference (LSD), the difference between the mean were considered significant at $P < 0.05$.

RESULTS

Egg hatch assay

The result show that the crude aqueous extracts of the experimental plants inhibited egg hatch of gastrointestinal nematodes at different concentrations as shown in Table 1. At concentrations of 500 ml/ml, poultice paste of *C. papaya* seeds and *T. vogelii* leaves and dried leaves of *T. vogelii* and *C. papaya* seeds showed efficacies greater than 95%. The LC_{50} for egg hatch inhibition were highest for dried and poultice paste of *C. papaya* seeds as shown in Table 3. Both dried and poultice paste of *C. papaya* leaves showed the lowest egg hatch inhibition among the extracts. Very low effects were recorded for distilled water control group. Increasing the concentration of the extracts caused a dose dependent significant ($P < 0.05$) decrease in egg hatch for all the extracts tested.

Laval development inhibition

The average efficacy of the decoctions to inhibit larval development is show in Table 2. The larval development inhibition of poultice and dried *C. papaya* and *T. Vogelii* were higher than 98% at concentration of 500 mg/ml. There was no significance difference between poultice and dried *C. papaya* and *T. Vogelii* for larval development inhibition at 500 mg/ml ($P > 0.05$). The minimum larval development inhibition was recorded for distilled water with a mortality rate of 1.3±4.03%. The LC_{50} for larval development are shown in Table 3.

Both poultice paste extract and dried extracts showed a dose dependent activity against both egg inhibition and larvae development inhibition for gastrointestinal nematodes. However, the overall performance of the dried extracts was better than that of the poultice paste extracts of the same extract.

DISCUSSION

This study demonstrates the existence of biologically active compounds with ovicidal and larvicidal effects in the plant extracts on gastrointestinal nematodes, even after heating for 1 h. The lower activity of dried and

Table 1. The mean inhibition of egg hatching ± SD for the different plant extracts compared to the distilled water negative control and Levamisol (3.125 mg/ml) positive control.

Concentration (mg/ml)	Egg hatching (%) ± SD					
	C. papaya leaves	*C. papaya* seeds	*T. vogelii* leaves	*T. villosa* leaves	Levamisol	Distilled water
Poultice paste (set 1)						
500	57.8±3.30	99.5±0.58	95.8±1.71	93.2± 0.9		
250	28.0±6.06	83.5±1.29	68.3±2.22	67.3±2.99		
125	16.0±4.97	72.3±4.57	52.3±4.19	55.5±4.44		
62.5	6.2±3.26	58.7±5.03	36.7+5.05	36.1±5.54		
31.25	3.4±2.19	37.8±4.11	24.9±4.12	21.3±2.10		
Dried (set 2)					100.00	1.3±2.03
500	59.5±3.42	99.0±0.82	95.8±1.71	87.0±3.16		
250	32.3±2.99	82.5±2.89	77.8±2.36	60.3±4.57		
125	19.8±5.06	75.3±3.78	66.3±3.60	47.8±2.99		
62.5	9.9±2.47	62.1±3.90	41.3±3.90	28.4±4.97		
31.25	6.9±2.62	35.5±4.79	29.7±6.88	19.9±4.29		

SD = Standard deviation

Table 2. The mean larval inhibition ± SD for the different plant extracts compared to the distilled water negative control and Levamisol (3.125 mg/ml) positive control.

Concentration (mg/ml)	Larval Inhibition (%) ± SD					
	C. papaya leaves	*C. papaya* seeds	*T. vogelii* leaves	*T. villosa* leaves	Levamisol	Distilled water
Poultice paste (set 1)						
500	63.0±2.58	99.5±0.58	99.0±1.41	81.8±2.99		
250	39.5±4.04	78.8±2.22	78.8±2.50	52.8±3.86		
125	23.3±3.59	71.8±2.50	69.3±2.63	41.0±3.65		
62.5	14.9±4.87	54.6±4.92	52.1±4.34	33.4±3.18		
31.25	5.1±4.21	42.1±3.11	35.6±3.92	22.4±2.99		
					100.00	1.3±2.03
Dried (set 2)						
500	60.1±2.08	98.8±0.50	98.3±0.96	86.8±4.35		
250	38.0±4.97	84.0±3.16	77.5±4.20	59.0±3.16		
125	20.3±3.78	78.5±1.83	68.5±3.11	46.0±2.45		
62.5	11.2±2.99	59.8±5.16	48.9±3.55	28.1±4.78		
31.25	9.3±5.40	47.9±5.23	40.5±6.18	17.6±6.10		

SD = Standard deviation

Table 3. LC_{50} and regression values for egg hatching and larval development of the plant extracts.

Preparation method	Plant extract	LC_{50} on egg hatching (LCL-UCL)	LC_{50} on Larval development (LCL-UCL)
Poultice paste (Set 1)	*C. papaya* leaves	431.32 (386.74-490.73)	335.00 (271.11-440.89)
	C. papaya seeds	49.94 (22.90-76.25)	49.32 (10.68-87.87)
	T. vogelii leaves	96.92 (49.37-167.26)	57.94 (24.15-92.80)
	T. villosa leaves	101.45 (65.98-148.51)	159.09 (83.59-321.35)
Dried (Set 2)	*C. papaya* leaves	417.83 (327.24-584.32)	386.54 (30.18-541.05)
	C. papaya seeds	48.81 (20.11-76.60)	38.36 (12.98-62.36)
	T. vogelii leaves	73.32 (60.61-86.63)	56.07 (16.73-96.80)
	T. villosa leaves	131.78 (110.40-157.90)	138.80 (116.84-165.92)

poultice paste of *C. papaya* leaves on egg hatching and larval development may be attributed to lack of ovicidal or larvicidal action of the metabolites or the alteration of these compounds by heating. Marie-Magdeleine et al. (2009) suggested that heating could potentially denature bioactive molecules, thereby influencing the anthelmintic activity of aqueous extracts of *Cucurbita moschata*. The study also shows that both the poultice paste extracts and the dried extracts of the plants evaluated showed a dose dependent egg hatching and larval development inhibition at tested concentrations. The probable reasons for the observed minor differences between the poultice paste extracts and dried extracts could be due to similarity of the solubility and the bioactive active constituents. Extracts from *C. papaya* seeds and *T. vogelii* leaves showed dose-dependent inhibition at lower concentration compared to other extracts.

In vivo studies have showed the potency of crude aqueous extract of *C. papaya* seeds against helminthes in sheep (Hounzangbe-Adote et al., 2001; Ameen et al., 2010) and goats (Fajimi et al., 2005) and poultry (Ameen, 2012). Incorporation of *C. papaya* leaves into goat feed resulted to increased feed intake and decrease egg per gram (EPG) in the feaces *in vivo* as well as *in vitro* (Daryatmo et al., 2010). Previous *in vitro* studies have showed anthelmintic effect of ethanolic extract of *C. papaya* seeds (Hounzangbe-Adote et al., 2005). Other studies on non-ruminants have also indicated potential anthelmintic effects of *C. papaya* latex and seeds on helminthes in mice, rats, pigs and poultry (Satrija et al., 1994, 1995; Sapaat et al., 2012; Bi and Goyal, 2012). The anthelmintic activities of *C. papaya* seeds extracts are associated with the presence Benzyl isothiocyanate (Kermanshai et al., 2001). Toxicity studies show that *C. papaya* seeds and leaves are considered safe for livestock and human consumption due to their low contents of oxalate and alkaloids compared to other commonly consumed food products (Adeniyi et al., 2009; Halim et al., 2011).

Crude aqueos extract of *T. vogelii* have shown significant activity against *Ascaridia galli* in indigenous chicken both *in-vitro* and *in-vivo* (Siamba et al., 2007). The methanolic leaf extracts of *T. vogelii* have also shown anthelminthic activity against *Nippostrongylus braziliensis* (Edeki, 1997). There is no scientific evidence of the *in vitro* or *in vivo* anthelmintic activity of *T. vogelii* or *T. villosa* extract in ruminants. However, their anthelmintic effect of these plants could be attributed to the presence of alkaloids, tannins, rotenoids and flavonoids constituents of the leaves (Marston et al., 1984; Ekpendu et al., 1998; Madhusudhana et al., 2010; Ahmad and Khan, 2013). Larvicidal and ovicidal effects of plants with these compounds against gastrointestinal nematodes have been reported in previous studies (Lateef et al., 2003; Siamba et al., 2007).

The extraction of plants in *in vitro* condition is not always comparable to those *in vivo* and as a result the outcome of the two assays can differ (Athanasiadou et al., 2001). *In vitro* tests only provide means for rapid screening for potential anthelmintic activities of plant extracts. The results therefore remain indicative and have to be confirmed through *in vivo* studies with experimental nematode infections in target host species. The potential of the plant aqueous extracts in this study in inhibit egg hatch and larval development may provide an alternative low-cost method for helminthes control, since the plants are available all-year round in Kenya.

Conclusion

Based on the result of this study, it can be concluded that *T. vogelii, T. villosa, C. papaya* leaves and seeds in form of crude aqueous extracts have anthelmintic activity in vitro against gastrointestinal nematodes of sheep. Based on the LC_{50}, the most potent decoction was that of *C. papaya* seeds for both egg inhibition and larval development inhibition. These studies suggest that these plant extracts could form an alternative to commercially available chemical anthelmintics drugs. In view of these findings, further research may be carried out for phytochemical screening and toxicity in order to exploit and verify the use of these plants as crude anthemintic agents. There is need to develop standardized methods for preparations for plants with good anthelmintic activity and formulate best alternative herbal preparation to replace or compliment the chemical anthelmintic drugs currently in use.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Review

Thinking out of the box: MADS-box genes and maize spikelet development

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Due to the importance of maize as an agricultural crop and its stature as an ideal model plant for the study of developmental biology in monocots, it is natural that research into its genetic structure has gained worldwide attention. Unfortunately, although much progress has been made in our understanding of the genetic control of the maize spikelet over the last decade, the depth of research in this field still lags behind that of dicots. Here, we review the developmental features of the maize spikelet and the characterization and function of MADS-box genes with the hope of stimulating further research in this area.

Key words: Maize spikelet, ABC model, MADS-box genes, regulation.

INTRODUCTION

Among the multitude of developmental phases in plants, flower formation is the most exciting and complex one. About 20 years ago, the ABC model for the genetic control of flower development was proposed and was initially based on the analysis of floral homeotic mutants in *Arabidopsis thaliana* and *Antirrhinum majus*. Later, the genetic regulatory network of flowering and floral meristem also began to be elucidated upon. Among these genes, transcription factors, especially MADS-box genes, play crucial roles in the whole regulation network (Krizek and Fletcher, 2005). Poaceae (grasses) is one of the most species-rich flowering plant families and includes many economically important crops. Flowers of grasses are arranged in spikelets, in which glumes, lemma, palea and lodicules are characteristic organs and serve as a basis for the classification of grasses (Schmidt and Ambrose, 1998). However, it still remains controversial whether they are equivalent to the bract, sepal and petal of dicot flowers. Thus, more research on the development of grass spikelets will provide a further understanding of the genetic control of monocot flower development and the molecular evolution of the grass-specific floral organs.

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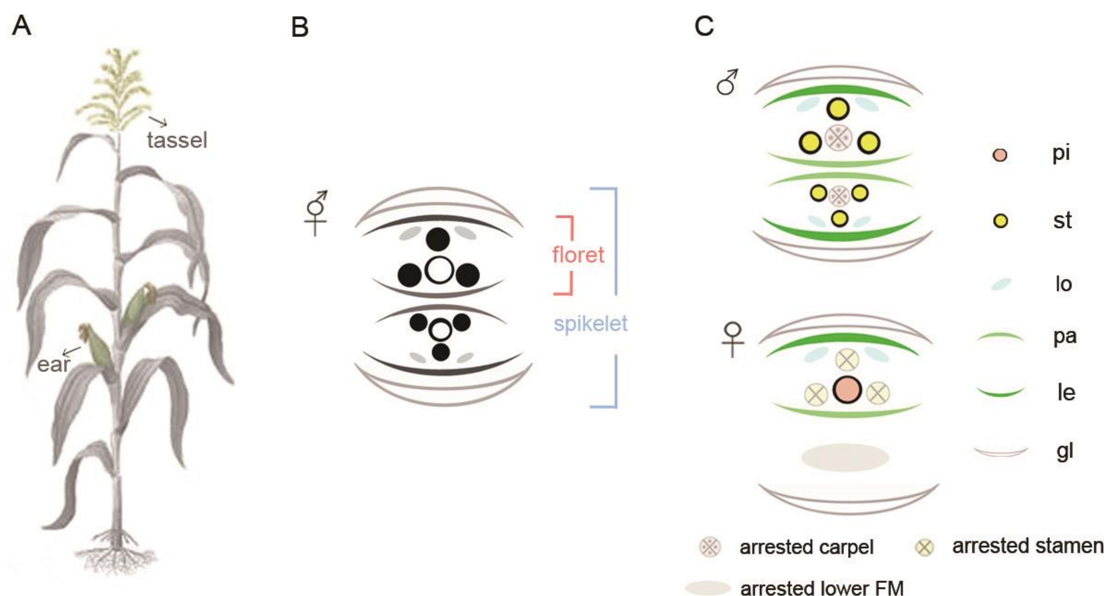

Figure 1. Maize plant and floral development. (A) Mature maize plant. **(B)** Maize floral development. At an early developmental stage, the spikelet is bisexual. **(C)** Mature spikelet. Later in development, pistils arrest in the floret of male spikelet (up) and the stamens arrest in the floret of female spikelet (below). **Pi,** pistil; **st,** stamen; **lo,** lodicules; **pa** palea, **le** lemma; **gl,** glume.

As one of the most important agricultural crops in the world, maize (*Zea mays* L. ssp. mays) is an important model plant for grass developmental biology because it possesses all of the following: a rich genetic history and abundant developmental mutants, a fully sequenced genome, and a good synteny with other grass species. Additionally, unlike rice producing hermaphrodite flowers, maize is a monoecious plant. Thus, research on the development of maize spikelets will contribute largely to understanding monocot plant flower development, the regulation mechanism of sex determination, and molecular evolution of the grass-specific floral organs. In this review, we summarize recent findings concerning maize spikelet development, mainly focusing on the characterization and functional study of MADS-box genes in maize flower organ development.

THE DEVELOPMENTAL STRUCTURE OF MAIZE SPIKELET

Maize is a monoecious plant that forms male and female inflorescence on a terminal tassel and on lateral ears, respectively (Figure 1A). At an early developmental stage, tassel and ear both initiate bisexual spikelets. Each spikelet contains two florets, the upper and the lower floret, which are subtended by two glumes. Each floret consists of a lemma, a palea, two lodicules, three stamens and a pistil (Figure 1B). Later in development, pistils cease to develop in the floret of male spikelets, and two unisexual florets form as a result of the pistil abortion; while in female spikelets, the lower floret and the stamens in the upper floret abort, resulting in each ear spikelet bearing a single female floret (Figure 1C). Therefore, unisexual flowers in maize are achieved by the process of selective arrest and abortion of the pistil or stamen primordium within a bisexual floret at the appropriate time (Thompson et al., 2009).

THE CHARACTERISTICS OF MADS-BOX GENES

MADS-box genes encode a family of transcription factors that play crucial roles in higher eukaryotes, especially in the regulation of floral development in flowering plants (Ciaffi et al., 2011). Previous phylogeny reconstructions revealed that the MADS-box gene family is composed of several defined gene clades. Almost all the plant MADS-box genes that are currently known are members of a monophyletic superclade of genes with a conserved structural organization, including a MADS (M-), intervening (I-), keratin-like (K-) and C-terminal (C-) domain, so called MIKC-type domain structure (Münster et al., 2002) (Figure 2). Some conserved motifs in the C-terminal domain play an important role in the formation of the MADS-box protein complex and transcription activation (Theissen et al., 1996). Many MIKC-type MADS-box genes that regulate floral development have been identified in angiosperms and can be divided into 12 major subfamilies by phylogeny reconstructions, including *AG, AGL6, AGL12, AP3*/*PI*, *GGM13* (*B_s*), *STMADS11*, *TM3*, *AGL2*, *AGL17*, *AP1*/*SQUA*, *AGL15* and *FLC*

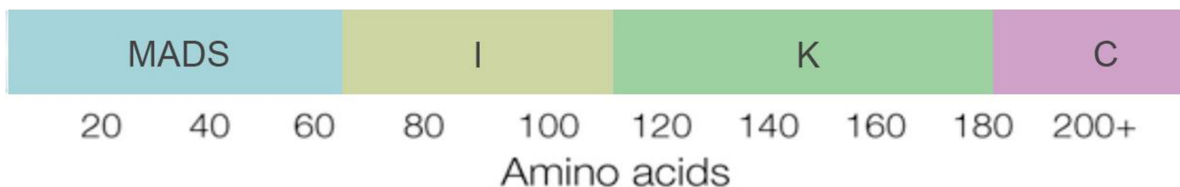

Figure 2. Domain structure of plant MIKC-type MADS-box protein.

(Becker and Theissen, 2003). So far, at least 32 MADS-box genes, belonging to 9 subfamilies, have been reported from maize and are widely involved in the regulation of floral organ identity, determinacy of the floral meristem, flowering time and the development of seeds (Table 1) (Zhao et al., 2011). Some maize MADS-box genes are functionally conservative with their orthologous genes in dicot plants, while the functions of others still await verification.

MAIZE HOMEOTIC MADS-BOX GENES

Studies on the two model eudicot plants *Arabidopsis* and *Antirrhinum* have led to the classic genetic ABC model that explains how three classes of genes (A, B and C) work together to specify floral organ identity. This model holds that A-class genes specify sepal fate in the first flower whorl, A plus B genes specify petals in the second whorl, B plus C genes give rise to stamens, and C genes alone are needed for carpel development in the fourth whorl (Coen and Meyerowitz, 1991). Later, the model has been expanded to incorporate D class genes, which are responsible for the development of ovules (Angenent et al., 1995), and E-class genes, which are necessary for the normal expression of A, B , C and D class genes and for the formation of functional complexes (Pelaz et al., 2000; Ditta et al., 2004). D class genes specify ovules, which will develop into seeds after pollination. Thus, some scholars define the model as “ABCDE model” (Goto et al., 2001). With the exception of the A-class gene *AP2*, all of those genes are members of the MIKC-type MADS-box family of transcription factors and they act by forming dimers and complexes of higher order. In *Arabidopsis*, there are two different class A genes, *APETALA1* (*AP1*) and *APETALA2* (*AP2*), two class B genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), one class C gene, *AGAMOUS* (*AG*) (Theissen et al., 2000), and one class D gene, *SEEDSTIK* (*STK*). The class E genes in *Arabidopsis* are represented by *SEP*-like genes (also known as AGL2 subfamily), namely *SEPALLATA1, 2, 3, 4* (*SEP1, 2, 3, 4*) (Ditta et al., 2004). Mutations of these genes will result in homeotic conversions of the regulated floral organs into organs of adjacent floral whorls, so these genes are also called homeotic genes. At present, the candidate class A, B, C, D and E genes from maize have been obtained by cDNA cloning, but it is still unclear whether the function of these genes is fully conservative with the homologous genes of dicotyledonous plants (Münster et al., 2002). Despite this, some important indications have been gained from the comparative functional analysis of rice orthologs (Cui et al., 2010; Kobayashi et al., 2010; Wang et al., 2010).

Putative A-class genes in maize

By cDNA isolation and phylogenetic sequence analysis, Münster et al. (2002) have found an orthologous relationship among *ZAP1* in maize, *AP1* in *Arabidopsis* and *OsMADS15* in rice. *OsMADS15* has been confirmed to control the differentiation of lemma and palea (Wang et al., 2010), two floral organs positionally orthologous to sepals, whereas until now only northern blot analysis gives us a limited clue of *ZAP1* as a putative A class gene that *ZAP1* was expressed in lemma, palea and lodicules, but not in anthers and carpel, similar with the expression pattern of *AP1* in *Arabidopsis* (Münster et al., 2002). Additionally, two other MADS-box genes, *ZMM4* and *ZMM15,* are orthologous genes of *AP1* and *OsMADS14* (Itoh et al., 2005). Because of the lack of the corresponding mutants, more supporting data is required before it can be determined whether or not these mentioned maize MADS-box genes have strict class A functions.

Putative B-class genes in maize

Compared to class A genes, the functions of class B genes have been proved to be conservative between monocots and eudicots. *Silky1* (*Si1*) in maize is an orthologous gene of *AP3* in *Arabidopsis*. *Si1* was expressed in anthers and lodicules. Compared to wild-type plants, *si1* mutants showed homeotic conversion of stamens to carpels and lodicules to palea/lemma-like structures, which suggest that palea/lemma in maize are homologous organs to sepals and lodicules are homologous to petals (Ambrose et al., 2000). Additionally, there are at least three class B genes in maize, *ZMM16*, *ZMM18* and *ZMM29*, which are orthologous genes of *PI* in *Arabidopsis* (Whipple et al., 2004). Whipple et al. (2004) have demonstrated that ZMM16 is capable of interacting *in vitro* with Si1, as well as with the orthologous

Table 1. MIKC-type MADS-box genes in maize.

Subfamily	Gene	Expression domain	Putative function	References
AG	*ZAG1*	Carpel, anther	*AG* orthologous gene	Schmidt et al., 1993
	ZAG2	Mature carpel	*STK* orthologous gene	Schmidt et al.,1993; Theissen et al., 1995
	ZMM1	—	*STK* orthologous gene	Theissen, 1995
	ZMM2	Anther	*AG* orthologous gene	Theissen, 1995
	ZMM23	—	*AG* orthologous gene	Münster et al., 2002
	ZMM25	—	*STK* orthologous gene	Münster et al., 2002
AGL2	*ZMM3*	—	*SEP-like* gene	Kobayashi et al., 2010
	ZMM6	Developing kernels and vegetative tissues	*SEP-like* gene	Lid et al., 2004; Kobayashi et al., 2010
	ZMM7	—	*SEP-like* gene	Fischer et al., 1995
	ZMM8	Upper floret meristem	*SEP-like* gene	Cacharrón et al., 1999; Kobayashi et al., 2010
	ZMM14	Upper floret meristem	*SEP-like* gene	Cacharrón et al., 1999; Kobayashi et al., 2010
	ZMM24	Spikelet meristem	*SEP-like* gene	Kobayashi et al., 2010
	ZMM27	Developing kernels and vegetative tissues	*SEP-like* gene	Lid et al., 2004; Kobayashi et al., 2010
	ZMM31	Spikelet meristem	*SEP-like* gene	Kobayashi et al., 2010
AGL6	*ZAG3*	Floral meristem, palea, lodicule, carpel	meristem gene	Becker and Theissen, 2003; Thompson et al., 2009
	ZAG5	Carpel	—	Becker and Theissen, 2003; Thompson et al., 2009
AGL17	*ZmMADS2*	Anther, pollen tube	Anther dehiscence	Schreiber et al., 2004
PI (DEF/ GLO)	*SILKY1*	Anther, lodicule primordium	*AP3* orthologous gene	Ambrose et al., 2000
	ZMM16	—	*PI* orthologous gene	Whipple et al., 2004
	ZMM18	—	*PI* orthologous gene	Whipple et al., 2004
	ZMM29	—	*PI* orthologous gene	Whipple et al., 2004
SQUA	*ZAP1*	Lemma, palea, lodicule	*AP1* orthologous gene	Münster et al., 2002
	ZMM4	Leaf primordia, young inflorescence	Early flowering	Danilevskaya et al., 2008
	ZMM15	—	—	Danilevskaya et al., 2008
	ZMM28	—	—	Münster et al., 2002
	ZmMADS3	Stem node, egg cell	Meristem gene, fertility	Heuer et al., 2001
STMADS11	*ZMM19*	—	*Tunicate1*	Han et al., 2012; Wingen et al., 2012
	ZMM20	—	—	Münster et al., 2002
	ZMM21	—	—	Münster et al., 2002
	ZMM26	—	—	Münster et al., 2002
TM3	*ZmMADS1*	Egg cell, central and antipodal cells	—	Heuer et al., 2001
Bs	*ZMM17*	Inflorescence, ovule	Reprodutive organs evolution	Becker et al., 2002

AP3. They have also shown that maize B-class genes are capable of rescuing the corresponding *Arabidopsis* B-class mutants, providing additional evidence of class B conservation (Whipple et al., 2004). Though there is still lack of studies on maize *PI*-genes related mutants, several lines of evidence from rice give us clues about their possible functions. Using RNAi and yeast two-hybrid strategy, *OsMADS2*, rice orthologous gene of *ZMM16*, has been proved to play an important role in lodicule and stamen development; in contrast, *OsMADS4*, orthologue of *ZMM18* and *ZMM29*, mainly takes part in stamen development (Prasad and Vijayraghavan, 2003; Yoshida et al., 2007; Yao et al., 2008). Thus, at least to some extent, we can make an easy speculation that, as putative

class B gene, duplicated *PI* clade MADS-box genes may function redundantly in maize stamen and lodicule development.

Putative C-class genes in maize

The typical class C gene of *Arabidopsis AG* is involved in controlling of floral determinacy and specification of carpel and stamen identity (Yanofsky et al., 1990; Coen and Meyerowitz, 1991). Such C gene activity may be diversified in maize because of gene duplication event. Maize contains two subclades of putative C-lineage genes, *ZAG1* and *ZMM2/ZMM23* (Kramer et al., 2004; Zahn et al., 2006; Dreni et al., 2007). The speculation about maize C function diversification is supported by the observations from their rice orthologous genes. A knockout line of *OsMADS3* (orthologous to *ZMM2/ZMM23*) and *OsMADS58* (orthologous to *ZAG1*) showed that both gene function as C-class genes. However, *OsMADS3* had a stronger role in specifying stamen identity and *OsMADS58* was more relevant in conferring floral meristem determinacy and in regulating carpel morphogenesis (Yamaguchi et al., 2006). Similarly, in the maize *zag1* mutants, floral meristem determinacy was partially lost, whereas stamens were almost normal in male flowers, suggesting that other class C genes, such as *ZMM2* and *ZMM23*, may be responsible for stamen specification. The expression pattern of *ZAG1* and *ZMM2* was consistent with this hypothesis; *ZAG1* is mainly expressed in carpels, while *ZMM2* is mainly expressed in anthers (Schmidt et al., 1993; Theissen et al., 1995; Mena et al., 1996). On the basis of these indications, *ZAG1* probably specifies floral meristem determinacy and *ZMM2/ZMM23* may specify organ identity of stamens, though mutants of *ZMM2/ZMM23* have not yet been identified.

Putative D-class genes in maize

Arabidopsis contains only one D class gene *STK*, which is involved in ovule development and seed dispersal (Pinyopich et al., 2003). By contrast, maize has three duplicated D class genes: *ZMM1* and *ZAG2*, together with rice *OsMADS13*, *Brachypodium BdMADS2* and wheat *TaAG-3*, belongs to one subclade, and *ZMM25* lies in another subclade with *OsMADS13*, *BdMADS2* and T*aAG-4*, according to phylogenetic analysis of D-lineage gene among grasses (Pinyopich et al., 2003; Paolacci et al., 2007; Ciaffi et al., 2011; Wei et al., 2013). Similar to *STK* gene, *ZAG2* primarily expressed in carple and ovule (Schmidt et al., 1993; Lopez-Dee et al., 1999). However, till now no more information have been gained about maize D class genes. Fortunately, recent studies on their grass counterparts may provide some interesting clues. Besides, expressing with a similar pattern with *ZAG2*, the *osmads13* knock-out mutant was completely female sterile, and its ovules were converted into a reiteration of ectopic carpels or into more amorphous structures with carpel identity (Dreni et al., 2007). Interestingly, knock-out of *osmads21* had a normal phenotype; moreover, *osmads21* could not modify the *osmads13* phenotype (Dreni et al., 2007). These data suggest that *OsMADS13* plays a role in ovule identity determination and floral meristem determinacy, while *OsMADS21* has probably lost this function during evolution (Dreni et al., 2007). In line with this, expression pattern and functional divergence have also been revealed among D lineage genes in wheat and *Brachypodium* (Paolacci et al., 2007; Wei et al., 2013). More or less, such divergence may also lie in the putative maize D class genes.

Putative E-class genes in maize

In *Arabidopsis*, class E genes function as cofactors with class A, B, and C genes, and in the absence of all four SEP genes, floral organs are transformed into leaf-like structures (Pelaz et al., 2000; Ditta et al., 2004). At least eight class E genes have been identified in maize and five in rice (Zahn et al., 2005; Arora et al., 2007; Cui et al., 2010; Ciaffi et al., 2011). Sequence analysis showed that *ZMM8* and *ZMM14* are orthologous to *OsMADS1* (*LHS1*) in rice (Cacharrón et al., 1999). *ZMM3* is orthologous to *OsMADS5*, *ZMM6* is orthologous to *OsMADS7*, *ZMM27* is orthologous to *OsMADS8*, and *ZMM24* and *ZMM31* are orthologous to *OsMADS34* (*PAP2*) (Kobayashi et al., 2010). *ZMM8* and *ZMM14* are found expressed in the all floral organs of the mature upper floret, but not in the lower floret. Cacharrón claimed that *ZMM8* and *ZMM14* work as selector genes to distinguish the upper from the lower floret during spikelet development (Cacharrón et al., 1999). In other words, *ZMM8* and *ZMM14* may be involved in conferring the identity or determinacy of the upper floret meristem, or they may prevent the conversion of the floret meristem into a spikelet meristem. *In situ* hybridization experiments revealed that *ZMM6* and *ZMM27* are not expressed during the vegetative growth period of maize, weakly expressed in the development of inflorescence, and strongly expressed during maize kernel development. But neither single mutant nor the *zmm6 zmm27* double mutant displays any obvious abnormities in kernel or flower development, suggesting that other SEP-like genes may provide functional redundancy with *ZMM6* and *ZMM27* (Lid et al., 2004) .

Rice has five SEP-like genes, *OsMADS1*, *OsMADS5*, *OsMADS57, OsMADS8* and *PAP2*/*OsMADS34*, whose functions are similar to SEP-like genes in *Arabidopsis* (Agrawal et al., 2005; Prasad et al., 2005; Cui et al., 2010; Kobayashi et al., 2010). However, it is still unclear whether the precise function of SEP-like genes in maize is conservative to the orthologous genes in *Arabidopsis*

or rice (Malcomber and Kellogg, 2004). Some research shows that the SEP-like genes in grass have a complex genetic lineage and a variety of expression patterns. These findings indicate that the SEP-like genes in grass are likely to undertake more function than their orthologous genes in *Arabidopsis* (Malcomber and Kellogg, 2004). However, it is still unclear whether the precise function of SEP-like genes in maize is conservative to the orthologous genes in *Arabidopsis* or rice (Malcomber and Kellogg, 2004). Some research shows that the SEP-like genes in grass have a complex genetic lineage and a variety of expression patterns. These findings indicate that the SEP-like genes in grass likely have additional functions to their orthologous genes in *Arabidopsis* (Malcomber and Kellogg, 2004).

PROSPECTS

Because of the agricultural and biological importance of maize, research on the roles of MADS-box genes in maize spikelet development has attracted worldwide attention. Although, research indicating that MADS-box genes regulate maize spikelet development has made great progress, it is still in the early stages. Many maize MADS-box genes were obtained by homologous cloning, but their expression patterns and function still remain unclear. The accumulating data from studies on MADS-box genes in dicots provide a guideline for the research of MADS-box genes in maize spikelet development. However, because of the unique floral structure of maize, whether or not the research of MADS-box genes in *Arabidopsis* can be applied to maize and other important crops needs to be verified by more powerful and diverse technologies. These could include isolation of maize mutants in related to MADS-box related genes, expression profiling of MADS-box genes, and the comparative study of their regulatory networks, and implementation of the maize Floral Genome Project (FGP). All of these studies will help to explore the function of MADS-box genes in maize and broaden our understanding of the molecular development and evolution of maize and other grass spikelets. Acquiring more understanding of MADS-box genes in maize spikelet development will promote awareness of the floral developmental mechanism of maize and other monocot plants.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Anti-*toxoplasma gondii* activity of constituents from *Balsamocitrus camerunensis* L (Rutaceae)

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Isolation, characterization and anti-*Toxoplasma gondii* activity of constituents from the CH_2Cl_2/MeOH (1/1) extract of the roots of the cameroonian plant *Balsamocitrus camerunensis* L. were investigated in this study. Four known coumarins derivatives were isolated, namely, marmin (1), imperatorin (2), xanthoxyletin (3), 6,7-Dimethoxycoumarin (4) and an acridone derivative namely 1-hydroxy-3-methoxy-acridone (5). Their structures were established on the basis of their spectroscopic data compared to reported results. Some of the isolated compounds showed noteworthy activity against *Toxoplasma gondii* intracellular parasite in mammals with an inhibition of parasite growth of around 46.44% for compound 4 and 82.12% for compound 3 which was the most active compound.

Key words: *Balsamocitrus camerunensis,* Rutaceae, coumarins, alkaloid, toxoplasmacidial activity.

INTRODUCTION

The discovery and development of anti-*toxoplasma* compounds is an effective way of controlling *Toxoplasma gondii* infections in humans and animals. *Toxoplasma gondii* is an obligate intracellular parasite that is able to infect a wide range of mammalian and avian species. In humans, *Toxoplasma* infections are widespread and can lead to severe disease in individuals with immature or suppressed immune systems. Consequently, *T. gondii* has become one of the major opportunistic infections of the AIDS epidemic (Saeij et al., 2005). Pregnant women without prior exposure to *T. gondii*, but that are infected during the first trimester of pregnancy can pass on the infection to the fetus resulting in abortion or serious neurological and eye pathologies of the baby at birth. (Remington and Klein, 1995)

The family Rutaceae consists of about 1500 species found principally in tropical and temperate regions (Meusel et al., 1978). Rutaceae species are used traditional medicine against elephantiasis, toothache, sexual impotence, gonorrhoea, malaria, dysmenorrhoea and abdominal pain (*Fagara zanthoxyloides)* (Kerharo and Adam, 1971; Ajanohoun et al., 1993; Anokbonggo et

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al., 1990; Watt and Breyer-Brandiwijk, 1962), Epigastric pain, vomiting, diarrhea, abdominal pain, colds, snake bites (*Zanthoxylum ailanthoides*), Aromatic tonic, stomachic and for fever, dyspepsia, cholera, Carminative and stomachic, used as a remedy for toothache, skin diseases, abdominal pain, anorexia, warm infestation, ataxia, Treat poisonous snake bites and also to treat diseases of the digestive (*Zanthoxylum alatum*), Treat fever, stomachache, flatulent colic, toothache and epilepsy (*Zanthoxylum capense*) (Medhi et al., 2013) etc. The chemistry of Rutaceae reveals the isolation of several secondary metabolites such as alkaloids, coumarins, flavonoids, phenylethanoids, lignans, limonoids, terpenoids, tannins and saponins, amongst others (Bouquet and Fouret, 1975; Wansi et al., 2009). Pharmacological studies on this family indicated many biological activities such as antitumor, antimicrobial, antifungal, controlling of cardiovascular and digestive system diseases (Lewis and Elin-Lewis, 1977; Metou et al., 1988; Sofowara, 1985). Previous phytochemical investigations of this genus revealed the presence of coumarins, quinoline alkaloids, free aliphatic acids and steroids, with some of these compounds exhibiting potent antibacterial, fungicidal, and algicidal properties (Tsassi et al., 2010). Plants of genus *Balsamocitrus* (including the decoction of the bark of *B. camerunensis*) are used in traditional African medicine to treat malaria, hypertension, infertility, and influenza (Tsassi et al., 2010; Asase et al., 2005).

As part of our systematic search for new bioactive lead structures from African medicinal plants, *Balsamocitrus camerunensis* L was selected for chemical and biological investigations. *B. camerunensis,* belonging to Rutaceae family, is a small tree of about 5 m high that is a newly discovered species found exclusively in Batouri (Cameroon) and Boukoko (Central African Republic) (Letouzey, 1963). Fouotsa et al. (2013) reported the presence of xanthones and coumarins in the stem bark of *B. Camerunensis*. To the best of our knowledge, there are no reported phytochemical studies on the roots of *B. Cameranensis*. Therefore, the objectives of this study were to isolate naturally occurring compounds from *B. camerunensis* roots and to evaluate their anti-*toxoplasma* activity. We report the isolation and characterisation of five known compounds isolated from the CH_2Cl_2/MeOH (1/1) extract of the roots of the plant *B. camerunensis*, namely marmin 1, imperatorin 2, xanthoxyletin 3, 6,7-Dimethoxycoumarin 4 and 1-hydroxy-3-methoxy-acridone 5, as well as their activity against intracellular *T. gondii* growth and proliferation.

MATERIALS AND METHODS

Sample collection

The plant *B. camerunensis* L. (Rutaceae) was collected from Dja (Batouri) in the East Province of the Republic of Cameroon, in January 2007 by Mr. Victor NANA of the National Herbarium of Cameroon who identified the plant. A voucher specimen is deposited at the National Herbarium, Yaounde, Cameroon.

Extraction and isolation

The air-dried and powdered roots (1.5 kg) of *B. camerunensis* L. was macerated with CH_2Cl_2/MeOH (1/1) at room temperature for 48 h. Removal of the solvent from the extract under reduced pressure yielded a yellow-brown residue (31.5 g). A part (30 g) of the crude extract was adsorbed on 40 g of silica gel with CH_2Cl_2/MeOH (1/1) as solvent and was submitted for gradient column chromatography on a 200 g silica gel column (type 60; 0.040-0.063 µm, Merck), using *n*-hexane-EtOAc, as the mobile phase and *n*-hexane as the eluent, resulting in the collection of 196 fractions (150 ml for each fraction). The 196 fractions were subsequently pooled into 11 major fractions (A-K) by combining the eluates on the basis of their mobility shift on TLC. Further separation of these fractions was done by repeated column chromatography using silica gel. Fraction A is the combined sub-fractions 1-11 (eluted with *n*-hexane- EtOAc 95:5) and fraction B (sub-fractions 12-26, eluted with *n*-hexane-EtOAc 9:1) was a mixture of fatty acids, hydrocarbons and phytosterols and was discarded. Fraction C (the combined sub-fractions 27-32, eluted with *n*-hexane-EtOAc 85:15) gave xanthoxyletin (3) (27.5 mg). From fraction E (composed of sub-fractions 37-42, eluted with *n*-hexane-EtOAc 85:15), we isolated imperatorin (2) (18.1 mg). Fraction G (composed of sub-fractions 62-71, eluted with *n*-hexane-EtOAc 75:25), was further separated by silica gel column chromatography, eluting with *n*-hexane-EtOAc in order of increasing polarity to yield marmin (1) (45.8 mg). From fraction H (the combined sub-fractions 72-75, eluted with *n*-hexane-EtOAc 75:25 and 70:30), we isolated HEN_4 6,7-Dimethoxycoumarin (4) (75 mg). Further purification of fraction J (the combined sub-fractions 82-86, eluted with *n*-hexane-EtOAc 65:35) gave HEN_5 1-hydroxy-3-methoxy-acridone (5) (97 mg). Fractions D, F, I and K were not further investigated.

Identification of compounds

The melting points were measured using a Büchi apparatus (Büchi melting points B-540) or on a microscope with heating platinum of Reicheirt, and are uncorrected. The optical activities were given at room temperature in methanol on a Perkin-Elmer 341 polarimeter. The α-Rotation of the light-polarized products dissolved in MeOH was measured in a 10 cm tank length. The D line (559 nm) of sodium was used as source of incidental light. The IR spectra were recorded using an infra red spectrophotometer in Fourrier Transformer of Nicolet 400 type on KBr pellet. Positions of the absorption bands were in cm^{-1}. UV spectra were recorded in MeOH solution on a Kontron-Uvikon 932 spectrophotometer. NMR experiments were carried out in various deutereted solvents (MeOH, acetone, $CDCl_3$) on a Varian Inova 500 (499.879 MHz) and a Varian Unity 300 (300.145 MHz) spectrometer for proton at 125.707 and for carbon spectra with TMS as internal standard at 75.087 MHz. Methyl, methylene and methine carbons were distinguished by APT (Attached Proton Test) experiments. Homonuclear ^1H connectivities were determined by using the COSY experiment. One-bond ^1H-^{13}C connectivities were determined with HSQC (Heteronuclear Single Quantum Coherence by 2D-multiple) gradient pulse factor selection. Two- and three-bond ^1H-^{13}C connectivities were determined by HMBC (Heteronuclear Multiple Bond Connectivity by 2D-multiple Quantum) experiment. Chemical shifts were reported in δ (ppm) and coupling constants (J) were measured in Hz. ESI mass spectra were recorded on a Quattro Triple Quadrupole mass spectrometer, with a Finnigan TSQ 7000 with nano-ESI API ion source. EIMS was performed on a Finnigan MAT95 (70 eV). High-resolution mass

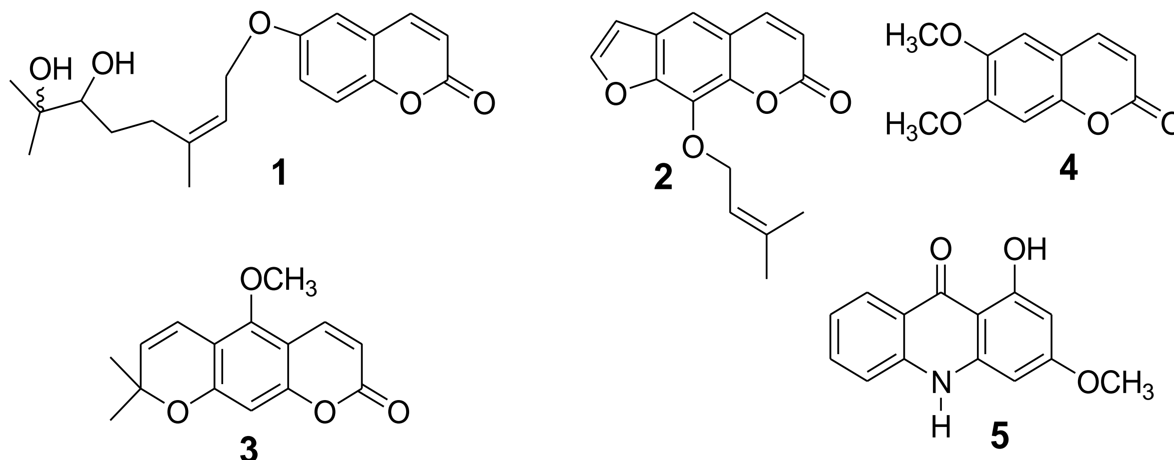

Figure 1. Structures of compounds 1-5.

spectra (HRMS) were recorded by ESI MS on an Apex IV 7 Tesla Fourier-Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Billerica, MA, USA). Column chromatography was carried out on variable diameters of columns by using like stationary phase silica gel with granulometry, 60 Merk between 70-230 mesh and 230-400 mesh. Thin-layer chromatography (TLC) was performed on Polygram SIL G/UV254 (Macherey-Nagel & Co.) and with pre-coated aluminium sheets silica gel 60 F254 TLC. Plates were used to check the purity of compounds. Spots were visualised by UV lamp (254 nm and 365 nm) or by 50% H_2SO_4/H_2O reagent. All reagents used were of analytical grades.

Assays of bio-activity

Cytotoxicity

The method of sulforhodamine B was used to assay the toxicity on embryonic fibroblastic MRC5 cells (Biomerieux, France) according to Fricker and Buckley (1996).

Quantification of Toxoplasma gondii by β-Galactosidase assay

Human foreskin fibroblasts (HFF) cells were infected by tachyzoites of *T. gondii* constitutively expressing *β*-galactosidase. The strain RH of *T. gondii* was used. The molecules to be assayed were added after 1 h of contact between *T. gondii* and HFF cells. *β*-Galactosidase assays were monitored at an absorbance of 540 nm/420 nm, to determine the parasite growth. The experiments were done in triplicate.

HFF cells, grown to full confluency in 96 well plates, were infected with 2×10^4 tachyzoites of genetically modified *T. gondii* constitutively expressing *β*-galactosidase. After one hour incubation of the parasites with HFF cells, the wells were washed with 200 µL of MEM medium, and 200 µL of fresh MEM medium containing dilutions of marmin (1), xanthoxyletin (3), 6,7-Dimethoxycoumarin (4) or 1-hydroxy-3-methoxy-acridone (5) added and incubated for 5 days in the presence of parasites. Two controls were made: uninfected and infected non-treated cells. The plates were centrifuged at 500 *g* for 5 min. The cells were lysed in HEPES 100 mM pH 8, MgSO4 1 mM, Triton X 100 1 %, DTT 5 mM buffer for 1 h at 50°C. Lysis was observed by microscopy. The reaction buffer (phosphate buffer 100 mM pH 7.3, *β* -mercaptoethanol 102 mM, $MgCl_2$ 9 mM) was added in each well, and the plate was incubated for 5 min at 37°C. Forty µL of chlorophenol red-*β*-D-galactopyranoside 6.25 mM in solution in phosphate buffer, pH 7.3, was added and the whole reaction mixture kept at 37°C until the appearance of a red coloration. The absorbance was measured at 540 nm/420 nm to determine parasite growth.

RESULTS

The powdered roots of *B. camerunensis* were extracted with the solvent system CH_2Cl_2/MeOH (1/1). The yellow-brown extract obtained was subjected to repeated column chromatography on silica gel, eluting with hexane followed by ethyl acetate in hexane with increasing polarity as mobile phase to obtain compounds 1-5 (Figure 1). These compounds were in agreement with data reported previously, identifying them as marmin 1 (HEN1), imperatorin 2 (HEN2) (Muller et al., 2004), xanthoxyletin 3 (HEN3), 6,7-Dimethoxycoumarin 4 (HEN4) (Perel'son et al., 1970) and 1-hydroxy-3-methoxy-acridone 5 (HEN5) (Spatafora and Tringaliw, 1997).

The compounds reported in this study marmin (1), xanthoxyletin (3), 6,7-Dimethoxycoumarin (4) and 1-hydroxy-3-methoxy-acridone (5) were evaluated on embryonic fibroblastic MRC5 cells for cytotoxicity using the sulforhodamine B assay (Fricker and Buckley, 1996) which gave an IC_{50} of 10 µg/mL.

We obtained an inhibition of parasite growth of around 5% for compound 1 (HEN1), 82.12% for compound 3 (HEN3), 46.44% for compound 4 (HEN4) and 22.03% for compound 5 (HEN5) (Figure 2).

DISCUSSION

Clinical treatment of toxoplasmosis in HIV-infected or AIDS patients has mostly been with atovaquone, a hydroxy-1,4-naphthoquinone, which is a structural analog

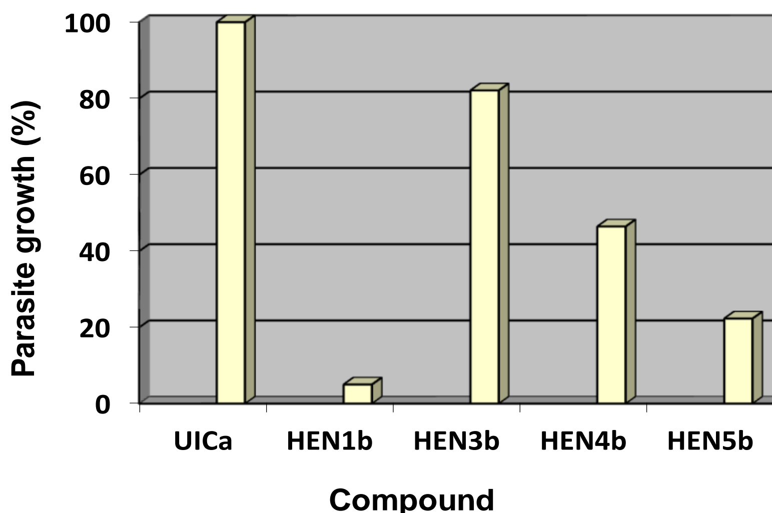

Figure 2. *Toxoplasma gondii* growth in presence of the different tested products. Parasites were incubated for 5 days in the presences of the products (in percentage). [a](UIC) Untreated infected cells. [b](HEN1) marmin, (HEN3) xanthoxyletin, (HEN4) 6,7-Dimethoxycoumarin and (HEN5) 1-hydroxy-3-methoxy-acridone.

of ubiquinone. *T. gondii* infection in immune-competent individuals often results in latent chronic infection, with parasite cyst formation in brain tissue. When the immune system of infected individuals wanes, such as in AIDS patients, the parasite is reacted leading to acute infection and toxoplasmosis clinical manifestation (Luft and Remington, 1992). CNS toxoplasmosis affects 7% of AIDS patients (Jones et al., 1999). Medications for immune-competent adults include pyrimethamine plus either trisulfapyrimidines or sulfadiazine. In pregnancy, spiramycin is usually given. Although these molecules are the drugs of choice for therapy and secondary prophylaxis of toxoplasmosis (Kovacs and Masur, 2000), some patients are intolerant to regimens. Thus there is urgent need to develop more effective and tolerable drugs against *T. gondii* infection. Therefore, the current study was conducted to develop a new anti-*Toxoplasma* agent from our local medicinal plant *B. camerunensis*.

The anti-*T. gondii* activity of *B. camerunensis* has not been reported before. In this study, we found that 6,7-Dimethoxycoumarin(HEN4) significantly inhibited *T. gondii* growth with an inhibition of parasite growth of around 46.44% (Figure 2). The most potent anti–*T. gondii* activity was found with xanthoxyletin (HEN3), which showed an inhibition of parasite growth of around 82.12% (Figure 2), indicating a good anti–*T. gondii* activity.

Similar results were also reported by Vardamides et al. (2008). They used two compounds isolated from the stem bark of *Turraeanthus africanus* to evaluate the anti-*T. gondii* activity on embryonic fibroblastic MRC5 cells for cytotoxicity using the same methods (sulforhodamine B assay which gave an IC_{50} of 10 µg/mL). The results show that Turraenthin (bezoic acid derivative) inhibited *T. gondii* growth of around 55% while Sesamin (lignane) inhibited *T. gondii* growth of around 20%.

The search for anti-*T. gondii* agent from Cameroonian medicinal plants has led to the finding that compounds isolated from *B. camerunensis*, in particular HEN3, showed potent anti-*T. gondii* activity. These results, first reported in this work, have allowed us to propose that *B. camerunensis* are likely the sources of new compounds that could be used to treat *T. gondii* infections. Further studies will be necessary to determine the toxicity of these active compounds and to purify the other fractions.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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Full Length Research Paper

Effect of three types of composts of olive oil by-products on growth and yield of hard wheat "*Triticum durum* Desf."

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The extraction of olive oil generates colossal by-products quantities; generally unexploited and causes serious environmental problems in Algeria. In tackling this problem, we performed three types of composts (C_1: 50% cows manure, 50% olive husks and olive mill wastewaters; C_2: 50% olive husks treated by the lime, 50% cows manure with fresh urea and olive mill wastewaters; C_3: 50% olive husks, 50% cows manure and water) for five months to use them as biofertilizers for hard wheat "*Triticum durum* Desf." 'Waha' cultivar. Results obtained showed that at the end of composting, the pH stabilized at 7.29 to 7.45; however saltiness was variable. For the three composts, the organic matter was degraded and the polyphenols content decreased significantly; C_2 was the compost that contains more mineral elements (N, P, K, Ca and Na). Indeed, the use of this compost as biofertilizer allowed an increase of the yield to 30.61% and an improvement of wheat growth, spikes' number (5.25±0.3 per plant in comparison with 1±0.09 for control) and seeds (57.12±0.99 per plant in comparison with 14.87±1.88 for control).

Key words: Compost, Olive husks, olive mill wastewaters, *Triticum durum* Desf., Algeria.

INTRODUCTION

Algerian olive cultures take the first place in fruit agriculture. In 2013, the olive production reached 5, 787, 400 qx, a part of this is intended for olive conserve (1, 749, 345 qx per year) and the rest for olive oil (4,038,055 qx per year) (Directorate of Agricultural Statistics (DSA), 2013); in fact, the production of olive oil had been 715, 970 hl.

The Technical Institute of Fruit Tree and Vine of Algeria (ITAF) recorded in 2013 an annual production of 497, 199 tons of olive oil by-products; 198, 880 tons of them are solid waste (olive husks) and 298, 319 tons liquid by-products called olive mill wastewaters. The production of olive oil is in increase in Algeria which lead to the rise of the by-products. The negligence of these later causes a great pollution.

The olive husks are rich in carbon and organic matter easily degradable, but because of their high content on

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Table 1. Composition of the three composts (C_1: compost 1; C_2: compost 2; C_3: compost 3).

Composts	Composition
C_1*	50% olive husks + 50% dry cows manure
C_2*	50% olive husks treated by the lime 0.5% + 50% cows manure with fresh urines
C_3	50% of olive husks + 50% of cows manure

*The final product was humidified by olive mill wastewaters diluted to 1/3 during the first month of composting and with plain water for the last 5 months.

polyphenols, they cause serious environmental problems (Alburquerque et al., 2004). Several researches were performed to exploit this waste in order to minimize pollution. The olive husks were treated and transformed into activated charcoal (Hemsas, 2008) or exploited for bio-combustion in order to produce electrical energy (Jaako et al., 2004). Also, they were used as cattle food, thanks to their nutritive values (Mennane et al., 2010).

Farmers historically have applied animal manure and human wastes to the land to increase their productivity (Nikus et al., 2004; Abebe et al., 2005). It was established that the organic matter portion of the soil is very important to maintain soil's physical and chemical properties optimum for crop production. Recently, extensive application of chemical fertilizers is becoming of increasing concern to the environment and human health (Fojlaley et al., 2014).

Therefore, using organic wastes like olive husks as fertilizers may reduce the amount of chemical applied to the soil; alternatively this can be achieved through other methods such as composting (Al-Widyan et al., 2005; Hachicha et al., 2006; Toscano et al., 2013; Gómez-Muñoz et al., 2013; Fernández-Hernández_et al., 2014), which is considered as an appropriate low-cost technology for organic waste recycling and organic fertilizer production (Antizar-Ladislao et al., 2006; Arvanitoyannis and Varzakas, 2008) and may well represent an acceptable solution for by-products disposal, adding value to this waste and enhancing the sustainability of the olive oil production system (Salomone and Ioppolo, 2011).

Because of the compact physical properties of olive husks due to their high content in lignin and fatty matter, saltiness, acidity and weak content of nitrogen (Alburquerque et al., 2004), it would be better to mix them with bulking agents in order to accelerate organic matter degradation; these substances can be manure, dead leaves and cereals straws (Garcia-Gomez et al., 2003; Alfano et al., 2008; Jenana et al., 2009).

Wheat is considered as one of the most important and strategic crops in the world. Algeria is the eight world's importer of these cereals; Technical Institute of Crop (ITC) imported 1.08 million tons of wheat in 2013 at 439 million dollars. Wheat culture have an agricultural importance in Algeria, which was cultivated at 1, 447, 902 ha in 2013 (ITC, 2013), but the production quality decreases and remains insufficient to reach local needs. There are several ways for increasing wheat production of which one of them is the appropriate application of organic residues (Yassen et al., 2010; Iqbal et al., 2014).

For this reason, it seems ingenious to use these huge amounts of by-products as organic fertilizer after composting. This study has an environmental interest, by the recuperation of waste for useful purposes, and an economical one by increasing yield and production of wheat and other species.

MATERIALS AND METHODS

Compost material

The olive husks and olive mill wastewaters were provided in November 2010 by a traditional olive oil extraction unit situated in the city of Sig (50 m altitude some 43 km, West North out of Mascara (West of Algeria) (35° 25′ 0″ N longitude; 0° 10′ 0″ E latitude). This region has Mediterranean climate. The cows manure was provided in November 2010 by a farm located in the suburbs of Mascara city.

Vegetal materials

We used hard wheat (*Triticum durum* Desf.) 'Waha' variety (Algerian) as test plant of the fertilizer value of composts. Wheat seeds were provided in October 2011 by the Technical crops Institute of the city of Saida (West of Algeria).

Composting

Composts were prepared in heap of 1 m high and 0.5 m width. Three types of composts were performed during five months; their composition is demonstrated in Table 1. An amount of 80 kg of composts (C_1; C_2; C_3) was each one mixed in tanks, and later placed on the soil; outdoors in nature. All composts were humidified with plain water during the process and covered with plastic film to preserve humidity. The humidification and ventilation were weekly.

During composting, the follow-up of temperature and pH was carried out every week.

Physicochemical analysis of olive husks and composts

Two hundred grams of olive husks (before composting) and the three composts were dried at 105°C and crushed to undergo the following physicochemical analyses:

pH and saltiness (electrical conductivity “EC”)

pH and saltiness (electrical conductivity “EC”) were measured according to the international method of 1/5 (Mathieu and Pieltain, 2003).

Dry material (DM)

DM was measured according to classical method by drying substrates at 105°C and calculated as seen in the following formulae (Mathieu and Pieltain, 2003):

Organic matter (OM)

OM was measured by substrates calcination in a muffle furnace at 850°C during one hour then calculated by classical method (Aubert, 1978).

Assimilable phosphorus

Assimilable phosphorus was calculated by Olsen method using blue molybdenum reactive and optic densities was read on the spectrophotometer at 860 nm (Olsen et al., 1954).

Total nitrogen

Total nitrogen was quantified according to Kjeldahl method by measuring the ammonia excess after organic matter mineralization (Van Reeuwijk, 1995).

Secondary elements (minerals)

Na, Ca and K were analyzed by a flame spectrophotometer (Pansu and Gautheyrou, 2003).

Organic carbon

Organic carbon was analyzed with Ann method by titration according to the diphenylamine principle (Dabin, 1967).

Total polyphenols

Total polyphenols were analyzed by Singleton and Rossi method (1965) according to the principle of Folin-Ciocalteu reactive and reading optic density on the spectrophotometer at 460 nm.

Use of composts

Five hundred seeds were chosen according to their size and quality and placed to germinate at 20°C in the dark. Ten days after, 100 seedlings were transplanted into 4 m^2 plots area containing soil of argilo slimy texture (Abad, 2009) and 3 kg composts (substrate 1 "S_1" = soil + C_1 ; substrate 2 "S_2" = soil + C_2 ; substrate 3 "S_3" = soil + C_3 and C “control” = soil). Seedlings were watered and the growth of stems length was followed up every week during six months. At the end of plants growth, we recorded the number of spikes and seeds and then calculated their weights per plant and for each substrate.

Statistical analysis

For physicochemical parameters, all data were analyzed in three replications. The experimental data were subjected to tests of variance analysis (ANOVA) at the risk of 5% and the obtained data were evaluated statistically using Student’s t-test; least significant difference was calculated at P<0.05 and signaled faces by letters (a, b and c) knowing that; P<0.05 significant difference “a”, P<0.01 very significant difference “ab” and P<0.001 highly significant “abc”.

RESULTS

Evolution of pH and temperature during composting

During composting, three phases of temperature and pH were distinguished. In the first phase, temperature and pH increased; however in the second one they decreased. During the last one, temperature was around 18-20°C and pH stabilized around 7.29-7.45 (Figure 1).

Physicochemical parameters

The physicochemical analyses results were shown in Tables 2 and 3. Physicochemical analyses were made at 5 and 6 months, but because of the slight difference between them, only those of 5 months are represented. In comparison to natural olive husks, the composted products have neutral to alkaline pH; an increase in saltiness of composts C_2 and C_3 and it decreases for C_1. The organic matter and polyphenols were more degraded in C_3 than C_1 and C_2 which was the richest in mineral elements (Na, Ca, K and N) and the most saline one.

Effect of composts on wheat plants growth

The statistical tests revealed that the growth of plants was significantly better in the substrates containing composts than the soil; S_1, S_2 and S_3 favored wheat plants to reach maximal length of stems (85.93 cm ± 1.75; 86.48 cm ± 1.68; 88.9 cm ± 1.23, respectively) compared to 71.8 cm ± 2.75 for the soil (Figure 2). Moreover, leaves had a bright green color and roots' ramification was dense.

The yield in number of spikes, seeds and weight was more important in the three substrates than the soil; we noted 36 ± 1.31; 57.12 ± 0.99 and 37.75 ± 1.03 seeds per plant for S_1, S_2 and S_3, respectively in comparison with 14.87 ± 1.88 seeds for control (Figures 3 and 4).

We noted an increase of the yield in weight of 1000 seeds of 16.32, 30.61 and 26.53% to S_1; S_2 and S_3 respectively in comparison with control. The yield in number of spikes of plants grown in S_1 was less than S_2 and S_3; but in seeds number, it was almost the same as S_3 (36 ± 1.31; 37.75 ± 1.03 seeds per plant for S_1; S_3, respectively) (Figure 3).

The yield in number of spikes was significantly better for plants grown in S_2 than those of S_3 (5.25±0.3 and 4.37±0.7 spikes per plant for S_2; S_3, respectively), and the number of seeds (57.12 ± 0.99 and 37.75 ± 1.03 seeds for S_2 and S_3, respectively) (Figure 3).

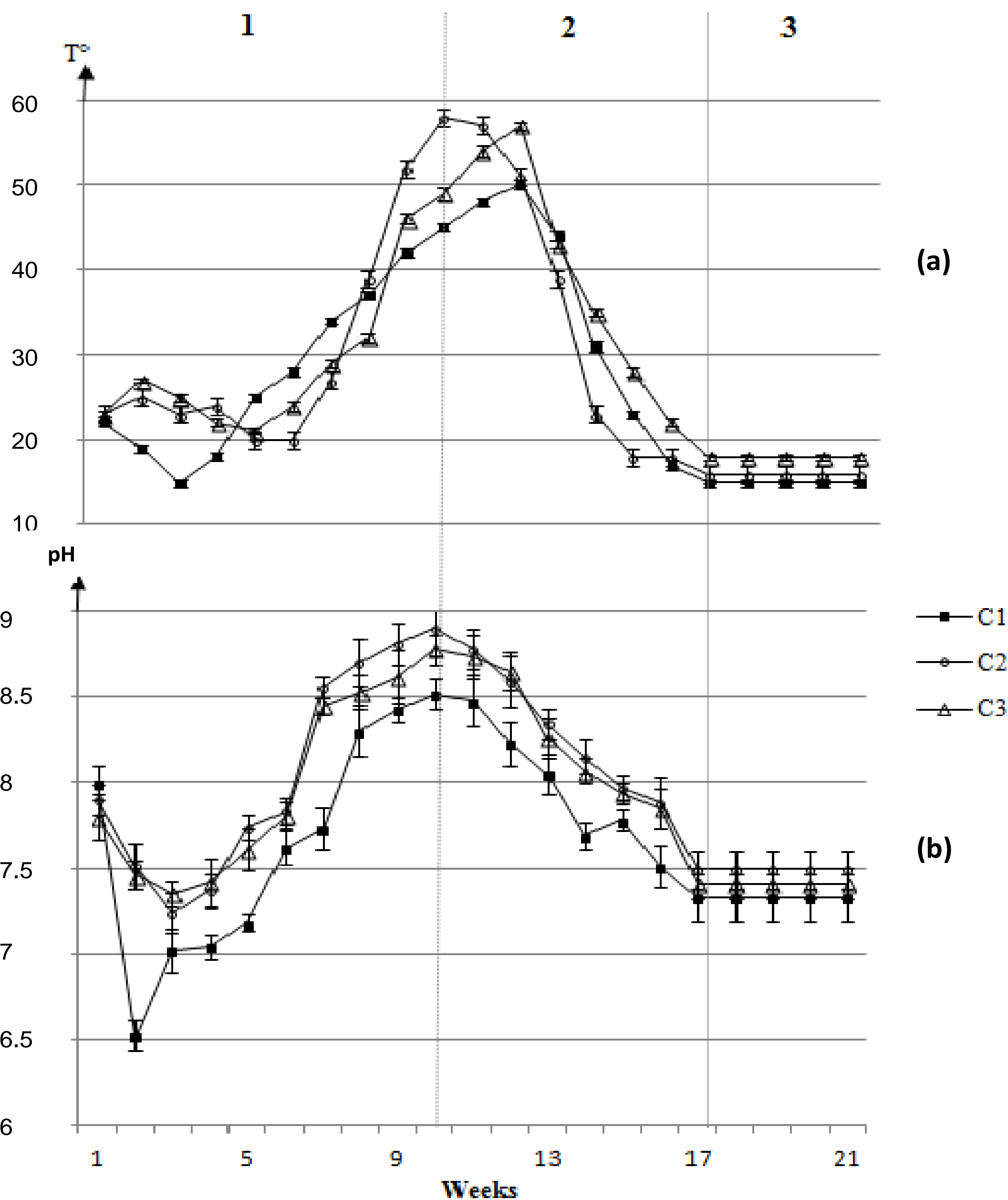

Figure 1. Evolution of temperature (a) and pH (b) of the three composts during the process (C_1: 50% cows manure + olive husks 50% + olive oil wastewaters ; C_2: 50% Olive husks treated by the lime + 50% cows manure (with fresh urea) + olive oil wastewaters; C_3: 50% Olive husks + 50% cows manure + water; "1": phase 1; "2": phase 2; "3": phase 3)

Table 2. Results of physicochemical analyzes (EC, Electrical conductivity; DM, Dry matter; OM, Organic matter) of the olive husks (oh), the three composts (C_1; C_2; C_3) and the soil (C).

Samples	pH	EC (µs/cm)	DM (%)	OM (%)	Polyhenols (mg/g)
Oh	6.17±0.04	1492.66±1.52	44.66±2.08	93.30±0.55	4.28±0.06
C_1	7.30±0.04	151.33±1.52	17.68±1.49	26.49±0.62	1.19±0.04
C_2	7.29±0.06	2580±2.08	16.12±1.23	21.20±0.38	1.07±0.05
C_3	7.45±0.08	3850±1	9.48±0.61	20.14±0.69	0.95±0.07
C	7.16±0.05	165±3.51	5.65±1.10	2.63±2.40	0.01±0.003

Table 3. Results of carbon (C) and minerals (Na, Sodium; K, Potassium; P, Phosphorus; N, Nitrogen) analysis of the olive husks (oh), the three composts (C_1; C_2; C_3) and the soil (C).

Samples	C (%)	Na (mg/g)	Ca (mg/g)	K (mg/g)	P (µg/g)	N (mg/g)
Oh	54.12±0.20	52±0.83	2.4±0.53	140±0.45	66.4±0.48	0.4±0.06
C_1	15.37±0.25	14±0.77	3.2±0.23	10±0.69	51.2±0.36	0.6±0.08
C_2	12.3±0.36	54±0.55	4±0.22	160±0.15	251.2±0.66	0.88±0.04
C_3	11.68±0.10	120±0.25	6.2±0.32	260±0.93	237.6±0.53	1.22±0.07
C	1.53±0.06	4±0.30	2.6±0.45	2±0.25	16±0.37	0.2±0.03

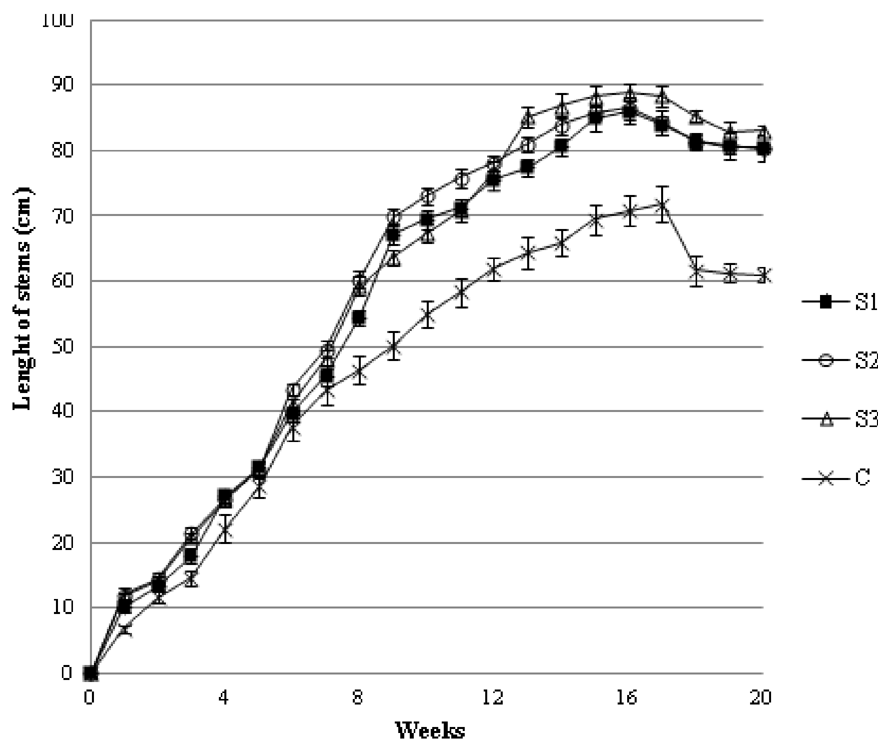

Figure 2. Evolution of wheat plants (S_1: Substrate containing soil + 3kg C_1; S_2: Substrate containing soil + 3kg C_2; S_3: Substrate containing soil + 3kg C_3; C: Control "soil": the vertical bar indicates the standard deviation of the mean of 100 plants per substrate.

DISCUSSION

Three pH and temperature changs have happened during composting. This agrees with many authors results (Vlyssides et al., 1996; Hachicha et al., 2008). The first is a mesophilic phase that corresponds to the high microbial activity ensuring an important production of heat (De-Viron, 2000).

The second is a thermophilic phase where only heat-resistant bacteria are present to degrade the organic matter transformed into CO_2 (Mustin, 1987). At the processing end, the temperature of composts decreased and the total polyphenols become stable which indicates the end of composts degradation and maturation (Kapetanios et al., 1993). At this stage, the humification predominates giving a stable product, mature and rich in humus (Mondini et al., 2004). At the end of composting, pH decreased to stabilize at 7.29 to 7.45.

We found a high salinity of compost humidified by olive mill wastewaters during the mesophilic phase. This is due to the degradation of organic components and liberation of olive mill wastewaters soluble salts (Paredes et al. 2001); though mineralization of organic matters during composting also participates in salinity increase. Our results are close to those obtained by Vakili et al. (2012).

We had noted a significant difference of the electrical conductivity between composts. Even if C_1 had been humified with olive mill wastewaters, an EC lower than C_3

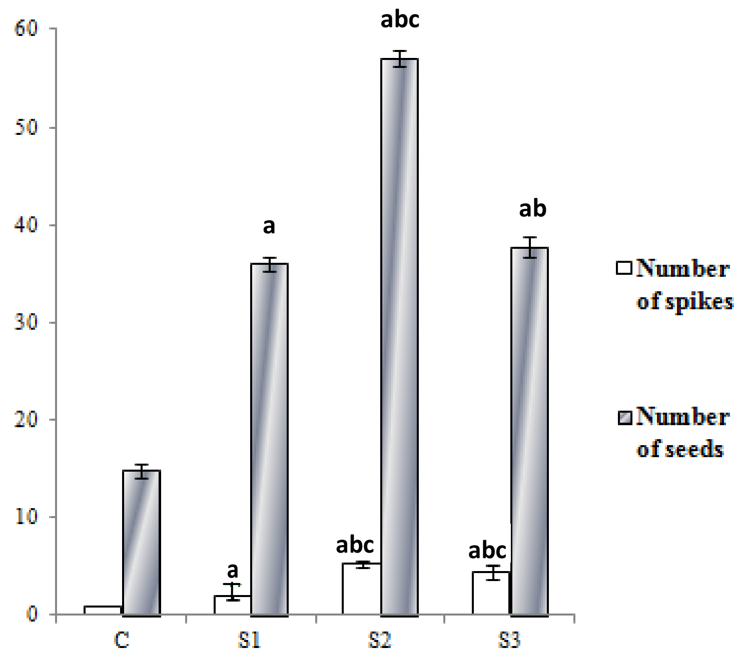

Figure 3. Number of spikes and seeds per plant of wheat (P< 0.05 significant difference “a”, P <0.01 very significant difference “ab” and P < 0.001 highly significant “abc”).

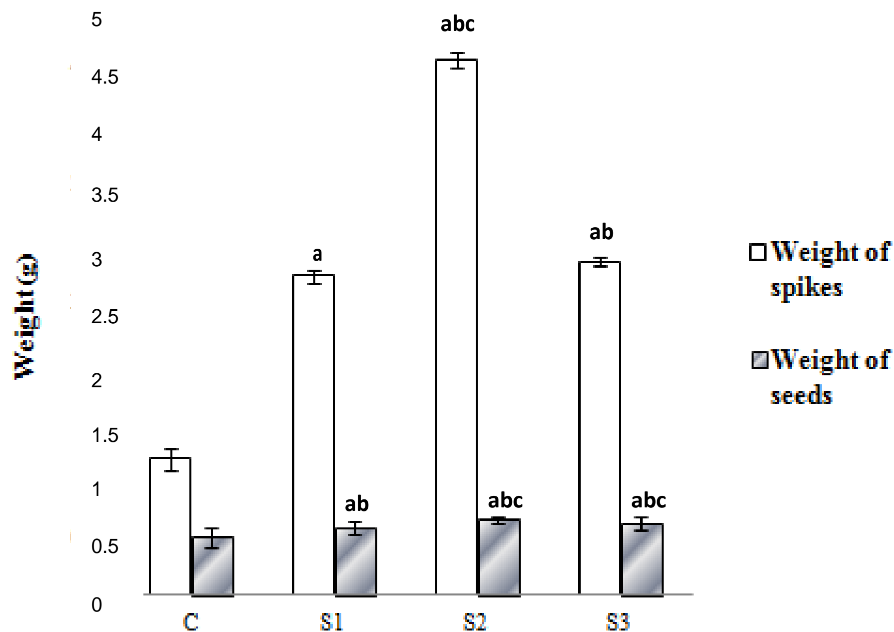

Figure 4. Weight of spikes and seeds per plant of wheat (P< 0.05 significant difference “a”, P<0.01 very significant difference “ab” and P < 0.001 highly significant “abc”)

was produced (151.33±1.52; 3850±1 µs/cm,respectively); this can be explained by a low organic matter degradation of C_1 which led to a lower release of salts compared to C_3.

At the end of the process, organic matters decreased. This can be explained by the mineralization phenomena. Our results are similar to those obtained by Sangaré et al. (2002) concerning compost of faeces, leftovers and urine

from sheep and results of Hachicha et al. (2009) studies of olive mill wastewater compost sludge and poultry manure and Michailides et al. (2011) about compost of olive husks and olive leaves. The organic matter was significantly more degraded in C_2 and C_3 (21.20%±0.38; 20.14%±0.69, respectively) than C_1 (26.49%±0.62); this is due to the composition of these milieus (C_2 and C_3) which favored an adequate microbial activity.

On the other hand, for the C_2 the addition of the lime in this compost had accelerated the degradation of the lignified texture of olive husks. This has been shown by Garcia-Gomez et al. (2003) about olive husks and cotton waste compost; Sellami et al. (2008) about compost of olive husks, poultry manure and sesame shells.

In comparison with natural olive husks, carbon content in composts was low and the nitrogen was increased; because during composting, microorganisms use the carbon to degrade the organic substance and release nitrogen.

Our results reveal that there are significant differences in the content of carbon and nitrogen between composts; notably, more important values in nitrogen and maximum reduction in carbon that is, C_2 and C_3. These were in correlation with the results obtained by Bernal et al. (1998) about compost of olive mill wastewater and many solid wastes; Hachicha et al. (2006); Hachicha et al. (2009) and Tortosa et al. (2012) about compost of olive mill waste and poultry sheep manure. For C_2, the increase of nitrogen is due to the addition of the urea in the milieu (Sangaré et al. 2002).

The C_3 have more content in nitrogen than C_2 (1.22 mg/g±0.07; 0.88 mg/g±0.04 for C_3 and C_2, respectively), this can be explained by the presence of urea in this later (C_2), this substance is acidifying and when mixed with plant wastes (manure and residue), it stimulated bacterial activity, resulting in a significant loss of nitrogen as ammonia.

During composting, the C/N had decreased and this indicate the maturity of compost (Abdelhamid et al., 2004) This is in correlation with the results of Vlyssides et al. (1996) about solid residue and wastewaters and Vakili et al. (2012) about compost of palm oil bio-wastes and poultry litter.

About the phosphorus, the result showed an increase in C_2 and C_3 (251.2 µg/g±0.66;237.6 µg/g±0.53, respect-tively) in comparison with olive husks in its natural state (66.4 µg/g±0.48).

The final composts contain higher concentration of secondary mineral elements (Ca, K, Na); a significant increase was noted in C_2 and C_3 minerals in comparison with the olive husks before composting and C_1. Ben Jenana et al. (2009) also noted an increase in these elements in a compost of posidonia, chicken manure and solid fraction of olive mill residues.

The final composts content in polyphenols decreased significantly in comparison with olive husks before composting (4.28 mg/g ± 0.06 to 0.95 at 1.19 mg/g) and ccording to Echeverria et al. (2011), it occurs due to the degradation of phenolic compounds during process in humic acid-forming.

Results reveal that S_2 marked the best developments of wheat plants and better yield in number of seeds (57.12±0.99) than the control (14.87±1.88) and in weight of seeds (0.644 g±0.035; 0.496 g±00.8 for S_2 and soil, respectively), also in number of spikes (5.25±0.3; 1±0.09 for S_2 and soil, respectively) and in their weights (4.54 g±0.07; 1.16 g±0.09 for S_2 and soil, respectively). Thanks to their high contents of mineral elements; the manure and olive mill wastewaters improved the nutritious quality of olive husks and increased their fertilizing ability. Indeed, Sellami et al. (2007) and Hachicha et al. (2008) reported that the compost of olive husks and manure, irrigated by olive mill wastewaters favours a better growth and yield of potato, as well as Jenana et al. (2009) results, on the compost of olive husks with the Posidonia and chicken manure on the tomato.

We noted that the development of wheat plants Waha cultivar was distinctly better in substrates containing composts than the soil (control), due to the presence of humic substance in composts which improve the plants growth and the mineral elements concentration.

Few studies have been published about wheat growth on olive husks compost, however we noted the effect of other types of composts on wheat growth such as Eusuf Zai et al. (2008) who tested compost with pea residue and chicken manure on a wheat cultivar: “Norin 61” and noted an improvement of growth and yield; same results were obtained by Ahmed et al. (2008) about “Inqlab 91” cultivar with a compost of fruit and vegetables waste.

Other data revealed that the yield in number of spikes, seeds and their weights was also better in substrates containing the compost than the control. This can be due to a good mineral nutrition. Notably, for S_2 a significant increase of the yield at 30.61% in weight of 1000 seeds in comparison with the control, these results are better than those found by Sefidkoohi et al. (2012) who reported an increase of 26.4% in weight of 1000 seeds of wheat cultivar ‘N-81-19’ using 40 t/ha of solid wastes compost + 1/2 NPK in comparison with the control. It has even been recommended that Hafidi et al. (2012) have shown an increase in the seeds yield of wheat cultivar ‘Marchouch’ of 111% in comparison with the control, using 12.8 kg of compost (based on sludge + green waste) in $4m^2$ area. It can be more interesting to increase the amount of compost that we produced for better increase of wheat yield.

Conclusion

Results obtained show that the composting of by-products improves nutritive quality. Final products had neutral pH and high saltiness. Organic matters were degraded and mineral elements content (N, P, K, Ca) increased but

polyphenols decreased. Substrate containing soil and compost made of olive husks treated by the lime, cows manure with fresh urines and olive mill wastewaters ensured best development of wheat plants and improve the yield.

The compostage of these wastes and their transformation in organic fertilizers is an interesting alternative for improvement of wheat production, minimization of importation of this one and valorization of olive oil by-products ensuring sustainable development irrespective of the environment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Screening and detection of extracellular cellulases (endo- and exo-glucanases) secreted by filamentous fungi isolated from soils using rapid tests with chromogenic dyes

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The screening plate method is commonly used for previous detection of cellulases produced by micro-organisms with biotechnological potential. In this manuscript, the authors aim to evaluate the hydrolytic ability of different fungi isolated from soil for the production of cellulolytic enzymes for cellulose degradation and determining the enzymatic index (EI) in relation to the growth of fungal colony and halo. The fungi were grown in carboxymethyl cellulose medium (CMC 1% w/v) and Avicel medium (Cellulose microcrystalline 1% w/v) for the determination of endo-glucanases and exo-glucanases respectively at 28°C for 48 h. Four chromogenic dyes were used: Congo Red, Phenol Red, Trypan Blue and Gram's Iodine. Also, another screening method was compared using carboxymethyl cellulose medium (CMC 1% w/v) at 28°C for 96 h and exposed with Congo Red dye in buffer Tris HCl 0.1 M, pH 8.0. The results obtained allowed to find significant differences between the tested fungi, the growth time and chromogenic dyes. The strains with higher Enzymatic Index (EI) were JCO1, UFT1, UFT2 and UFT3 for endo-glucanases and JCO2, UFT1, UFT2 and UFT3 for exo-glucanases.

Key words: Cellulases, chromogenic dyes, filamentous fungi, endo-glucanase, exo-glucanase.

INTRODUCTION

In every year, millions of tonnes of waste of lignocelluloses formed by cell walls incorporated mainly of cellulose, being one of the compounds most abundant and hard-to-degrade in nature are generated (Sae-Lee and Boonmee, 2014); and are present in different sources such as agricultural, industrial, forestry and agro-industrial waste; becoming attractive feedstocks for the generation of different by-products (Gupta et al., 2012). Most of these residues are burned in the open, generating dioxins due to the combustion conditions and affecting the environment, human and animal health (CEC, 2014).

Cellulose is a polysaccharide constituted by crystalline structures comprised of chains of β(1–4)-D-glucose,

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recognized for its potential for energy generation and with recent studies for the production of second generation ethanol (Glass et al., 2013; Johnsen and Krause, 2014). However, to obtain these by-products, cellulose must be subjected to enzymatic degradation process with the help of a system that allows conversion into glucose units. Within enzymatic processes are included biological systems developed by different microorganisms such as bacterial and fungal species (Chakraborty and Mahajan, 2014). The cellulases produced by fungal species are formed by a cellulolytic complex of endo-β-1,4-gluca-nases (EC 3.2.1.4), exo-β-1,4-glucanases or cello-biohydrolases (EC 3.2.1.91) and β-glucosidases or cellobiases (EC 3.2.1.21) (Szakacs et al., 2010). The endo-glucanases are responsible for degrading cellulose chains internally, where at the same time, the cello-biohydrolases hydrolyze the reducing and non-reducing ends releasing cellobiose and finally these are hydrolyzed by glucosidases in glucose molecules (Gilbert, 2010; Glass et al., 2013). Therefore, the native fungal cellulases isolated from various sources such as soil and decaying wood are commercially important for their resistance to high temperature conditions, pH changes and by high levels of enzyme secretion (Juturu and Wu, 2014).

The screening method for the detection of extracellular enzymes using dyes developers or chromogenic such as Congo red, Phenol red, Trypan blue, Gram's iodine, Remazol brilliant blue, it has been commonly used for *in vitro* selection of polysaccharides degraders microorganisms and characterized as a simple, fast and cost-efficient technique (Yoon, et al., 2007; Kasana et al., 2008; Jo et al., 2011). Where, the cellulolytic activity is reflected by the appearance of clear halos that surround the colony and not degraded areas arise without exposure or color variation (Johnsen and Krause, 2014).

These previous tests applied in laboratory scale fermentations are important in the selection of indicator strains of cellulolytic activity, where the lignocellulosic waste are degraded and recovered by the same industries such as the production of bioethanol and paper pulping. This study aimed to evaluate the enzymatic capacity of different filamentous fungi isolated from soil for the production of extracellular cellulases using different chromogenic dyes (congo red, phenol red, trypan blue and gram's iodine). In this case, the enzymatic index calculated by colony growth in relation to enzymatic halo released by the endo-glucanases and exo-glucanases used as carbon sources: carboxymethyl cellulose (CMC) and cellulose microcrystalline (Avicel) respectively will be analyzed.

MATERIALS AND METHODS

Fungi strains

Eight fungi strains granted by the Microbiology Laboratory located in Biotechnology-Based Business Incubator of the Federal University of Tocantins, Gurupi Campus, Brazil were employed. Where, JCO1, JCO2, JCO3, JCO4 and JCO5 belong to the company JCO Fertilizers and were isolated from soils in Barreiras Municipality, Bahía, Brazil. The fungi UFT1, UFT2 and UFT3 were isolated from soils by Federal University of Tocantins, Gurupi Campus, in the town Lagõa da Confusão, Tocantins, Brazil. The strains were replicated and stored in PDA medium (Potato 200 g/L, dextrose 20 g/L, agar 15 g/L). The fungi grew at 28°C for 8 days and preserved at 4°C for 3 months.

Screening and evaluation of cellulases enzymatic activity

To determine production of Endo-1,4-β-D-glucanase, the strains were grown in CMC medium (Carboxymethyl Cellulose 1% w/v), adapted from Kasana et al. (2008), as sole carbon source (%w/v): 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 1% carboxymethylcellulose sodium salt, 0.05% peptone and 2% agar. For the productions of Exo-1,4-β-D-glucanase (Avicelase), the strains were grown in Avicel medium (Cellulose microcrystalline 1% w/v) composed of the same salts as the CMC medium (Jo et al., 2011). The plates were inoculated with a fungal mycelium disc of 1 cm diameter and incubated at 28°C for 48 h in darkness (Agustini et al., 2012).

Extracellular cellulases detection using indicator dyes

The enzymatic activity colorations using indicators were: Congo Red, Phenol Red, Trypan Blue and Gram's Iodine (Vetec, Synth, Vetec, Newprov). After the incubation period, the first plates were flooded with 10 mL Congo Red (0.1% w/v) solution. After 30 min, the solution was discarded. The crops were washed with 5 mL NaCl (0.5 M) solution for 10 min (Teather and Wood, 1982; Kim et al., 2000). The second and third sets of plates were flooded with Phenol Red and Trypan Blue respectively (Yoon et al., 2007) and it proceeded in the same way as with Congo red solution. The last set of plates was flooded with 10 mL Gram's Iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water) for 3 to 5 min (Kasana, et al., 2008; Johnsen and Krause, 2014).

Comparison of the screening method using Congo Red

Also, it was compared and another screening method in plate described by Ruegger and Tauk-Tornisielo (2004) and proposed by Nogueira and Cavalcanti (1996) for the detection of Endo-1,4-β-D-glucanase was adapted: The strains were grown in carboxymethyl cellulose medium (CMC 1% w/v) as sole carbon source (%w/v): 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.001% $FeSO_4{\cdot}7H_2O$, 1% carboxymethylcellulose sodium salt and 2% agar. The plates were inoculated with a fungal mycelium disc of 1 cm diameter and incubated at 28°C for 96 h in photoperiod. After that period, they were grown at 37°C for 16 h, 10 mL of Congo Red (0.1% w/v) in Tris-HCl buffer 0.1 M and pH 8.0. After 30 min, the solution was discarded. The crops were washed with 5 mL of NaCl (0.5 M) solution for 10 min (Kim et al., 2000).

Molecular identification of strains

After screening assays, the strains isolated from JCO Fertilizers JCO1, JCO2, JCO3, JCO4, JCO5 that are part of the fungi mix of Trichoplus product were identified through the sequencing of the ITS region, in the Biological Institute, São Paulo, Brazil. The isolates UFT1, UFT2 and UFT3 still were unidentified molecularly, therefore were compared with respect to fungi identified. DNA extraction was carried out according to the methodology of CTAB

Table 1. Molecular identification of fungi isolated from the Trichoplus product of JCO Fertilizer.

Isolated	Identified species	GenBank access	% Similarity index
JCO1	*T. asperelloides* GJS04-217	DQ381958	100
JCO2	*T. longibrachiatum* DAOM 167674	EU280099	100
JCO3	*T. harzianum* CIB T44	EU280077	100
JCO4	*T. harzianum* CIB T44	EU280077	100
JCO5	*T. asperelloides* GJS 04-217	DQ381958	100

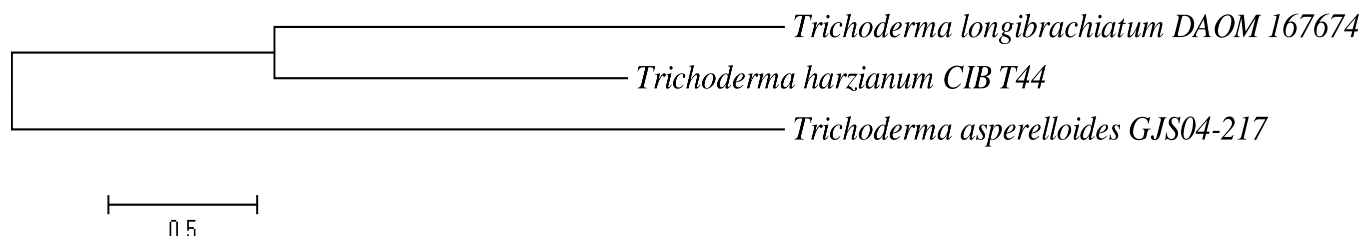

Figure 1. Phylogenetic tree analyzed using neighbor-joining method in the MEGA6 software.

(Cetyltrimethylammonium Bromide) described by Doyle and Doyle (1987). The polymerase chain reaction (PCR) for amplification of gene fragment encoding elongation factor (EF) was performed with the primer pair tef71F (5" – CAAAATGGGTAAGGAGGASAAGAC – 3") and tef997R (5" – CAGTACCGGCRGCRATRATSAG – 3") (product size of approximately 930 pb) (Shoukouhi and Bissett, 2008). The reactions were performed in thermal cycler PTC100 (MJ Research) according to the following schedule: initial denaturation at 94°C for 2 min, 40 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 60 s and final extension at 72°C for 4 min. The polymerase chain reaction (PCR) products were purified by precipitation with polyethylene glycol, according to the protocol described by Schmitz and Riesner (2006). The sequencing was conducted by chain termination method with the Big Dye 3.1 reagent (Applied Biosystems) in an automatic sequencer ABI3500 (Applied Biosystems). The sequences obtained were compared with sequences of voucher specimens deposited in the database of the ISTH International Subcommission on Trichoderma and Hypocrea Taxonomy). Identification of strains using molecular methods was carried out by sequences BLAST. The ITS sequences were retrieved from GenBank and the phylogenic trees were constructed using neighbor-joining method in the MEGA6 software (Tamura et al., 2013).

Data analysis

The enzymatic index was determined between the colony diameter and the enzymatic halo diameter (EI = Øh/Øc) (Herculano et al., 2011). The halo presence or clear area around the crop was the cellulase activity indicator evidenced by the enzymes secretion made by fungi through the culture medium (Sharma and Sumbali, 2014). The data was analyzed using IBM SPSS Statistics 19 by analysis of variance ANOVA and Tukey and Duncan multiple comparisons with a confidence level ($p = 0.05$).

The trials were conducted in triplicate and was performed in an experimental design for each of the dependent variables: Enzymatic Index (EI) of endo-1,4-β-D-glucanase and Exo-1,4-β-D-glucanase. Each design composed of three factors or independent variables: Strains, dyes and medium; with different levels or variations factor. In the case of 48 h of growth, the tests were performed in 8 × 4 × 1 factorial scheme per dependent variable. The second screening method was compared with respect to the average values obtained by the first method using CMC medium and Congo Red dye. The independent variables were: Strains, dyes, medium and time. Eight fungal strains, Congo Red dye with and without buffer, growth time 48 and 96 h, and two CMC medium described by authors with different methodologies were employed. The test was performed in 8 × 2 × 2 × 2 factorial scheme per dependent variable and in this case, for the detection of endo-1,4-β-D-glucanases.

RESULTS AND DISCUSSION

Five of the eight strains of filamentous fungi were identified molecularly (Table 1). The strains UFT1, UFT2, UFT3 were not yet sent for identification. The strains JCO1 and JCO5 were identified as the same species of *T. asperelloides* GJS04-217 and the strains JCO3 and JCO4 identified as *T. harzianum* CIB T44. Once identified molecularly, the three fungal species were analyzed using neighbor-joining method in the MEGA6 software (Figure 1). In the case of JCO1 and JCO5, the strains were in a separate group compared with the strains JCO2, JCO3 and JCO4. Therefore, T. longibrachiatum DAOM 167674 and T. harzianum CIB T44 showed a similar grouping. The results of screening methods were statistically analyzed by analysis of variance. The Fisher distribution test indicates a value of F-critical less than F-ratio for the CMC and Avicel medium (Table 2). Therefore, the Enzymatic Index Averages (EI) presents differences significant to the 1% of probability. In the second method with Congo Red (Figure 2), after 96 h of

Table 2. Analysis of variance of the experiment using the Fisher distribution test.

Source of variance	Sum of squares	Degrees of freedom (df)	Mean squares	F-ratio	F-critical
CMC ANOVA					
Between groups (Strains)	4.117	7	0.588	17.366**	2.849
Within groups (Dyes)	2.980	88	0.034		
Total	7.097	95			
AVICEL ANOVA					
Between groups (Strains)	7.511	7	1.073	38.321**	2.849
Within groups (Dyes)	2.464	88	0.028		
Total	9.974	95			

**Significant at 1% probability.

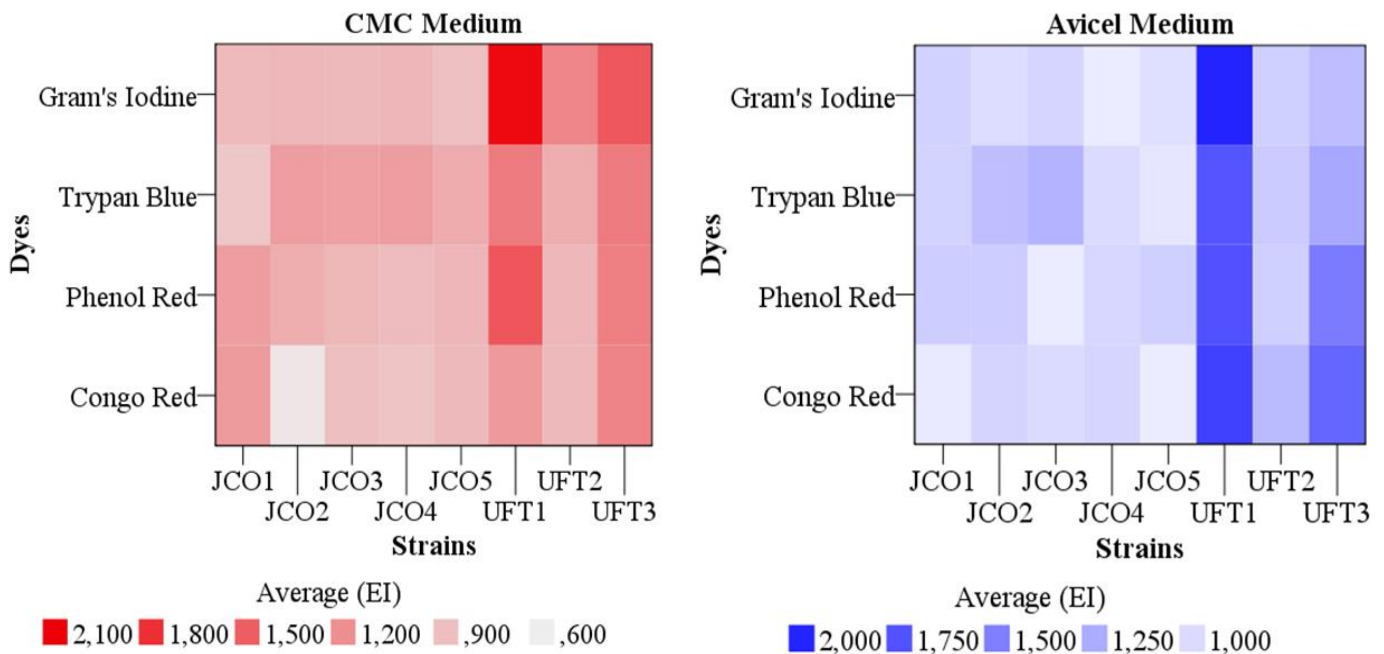

Figure 2. Statistical average group for the studied strains with different dyes in CMC and Avicel medium.*Second screening method for 96 h.

growth, some strains that were not evident development of halo at 48 h can be described as positive producers of Endo-glucanases as the strains JCO1, JCO3, JCO4 and JCO5. The enzymatic indices near 2.1 have better production of Endo-glucanases. In the case of Exo-glucanases, the fungi have a similar growth behavior with different chromogenic dyes. The enzymatic indices above 1.0 clearly represent enzyme secretion outside the colony and those with values below 1.0 defined the colony growth higher than the enzymatic halo.

Endo-glucanases production in CMC medium (Table 3 and 4) with 48 h of growth, the fungi strains revealed on Congo Red with more EI were UFT3, UFT2 and UFT1 with values of 1.269, 1.130 and 0.933, respectively. For the Trypan Blue dye, the best indices were in the strains UFT3, UFT1 and JCO2 with values of 1.327, 1.323 and 1.110, respectively. With Phenol Red dye, the best values were found in the strains UFT1, UFT3 and JCO1 with 1.450, 1.301 and 1.102, respectively. The growth evidenced in Gram's Iodine shows the highest rates to the strains UFT1, UFT3 and UFT2 with values of 2.024, 1.550 and 1.254, respectively. Comparing these with the second screening method with Congo Red at 96 h, the best indices were for the strains UFT1, UFT3 and JCO1 The similarities between the grouped strains can be explained once demonstrated by the molecular identification,

Table 3. Comparison of enzymatic index represented in CMC medium using ANOVA analysis of variance and post-hoc tests.

Strains	Congo Red	Trypan Blue	Phenol Red	Gram's Iodine	$\overline{TXEI}$	σ^2	$\hat{S}$	CV (%)	$S_{\bar{x}}$	HDS (p = 0.05)				
	$\overline{XEI}$									Tukey		Duncan		
JCO1	0.920	0.849	1.102	0.925	0.999	0.021	0.147	14.71	0.042	A		A		
JCO2	0.652	1.110	1.012	0.939	0.928	0.038	0.195	21.01	0.056	A		A		
JCO3	0.895	1.093	0.921	0.931	0.967	0.009	0.095	9.82	0.027	A		A		
JCO4	0.865	1.103	0.915	0.951	0.959	0.016	0.126	13.14	0.036	A		A		
JCO5	0.931	1.013	0.950	0.889	0.946	0.004	0.064	6.77	0.019	A		A		
UFT1	1.130	1.323	1.450	2.042	1.513	0.132	0.363	23.99	0.105		B		B	
UFT2	0.933	1.009	0.949	1.254	1.036	0.020	0.140	13.51	0.040	A		A		
UFT3	1.269	1.327	1.301	1.550	1.362	0.031	0.177	13.00	0.051		B			C
Significance										0.84	0.48	0.22	1.00	1.00

$\overline{XEI}$, Enzymatic index average; $\overline{TXEI}$, Enzymatic index total average; σ^2, Variance; $\hat{S}$, Standard deviation; CV, Coefficient of variation; $S_{\bar{x}}$, Standard error of mean; HDS, Homogeneous subsets; p, significance.

Table 4. Comparison of enzymatic index represented in Avicel medium using ANOVA analysis of variance and post-hoc tests.

Strains	Congo Red	Trypan Blue	Phenol Red	Gram's Iodine	$\overline{TXEI}$	σ^2	$\hat{S}$	CV (%)	$S_{\bar{x}}$	HDS (p = 0.05)					
	$\overline{XEI}$									Tukey			Duncan		
JCO1	0.923	1.042	1.074	1.045	1.021	0.017	0.132	12.93	0.038	A			A		
JCO2	1.038	1.152	1.076	0.989	1.064	0.007	0.083	7.80	0.024	A			A		
JCO3	0.997	1.215	0.903	1.030	1.037	0.034	0.185	17.84	0.053	A			A		
JCO4	1.030	0.998	1.021	0.906	0.989	0.004	0.067	6.77	0.019	A			A		
JCO5	0.906	0.935	1.061	0.977	0.970	0.007	0.085	8.76	0.025	A			A		
UFT1	1.847	1.733	1.756	2.006	1.835	0.084	0.289	15.75	0.084		B			B	
UFT2	1.181	1.085	1.057	1.057	1.095	0.014	0.117	10.68	0.034	A			A		
UFT3	1.647	1.275	1.522	1.162	1.401	0.056	0.238	16.99	0.069			C			C
Significance										0.60	1.00	1.00	0.11	1.00	1.00

$\overline{XEI}$, Enzymatic index average; $\overline{TXEI}$, Enzymatic index total average; σ^2, Variance; $\hat{S}$, Standard deviation; CV, Coefficient of variation; $S_{\bar{x}}$, Standard error of mean; HDS, Homogeneous subsets; p, significance.

where the strains JCO1, JCO2, JCO3, JCO4 and JCO5 belong to the same fungal genus. However, the enzymatic indices of unidentified isolates were higher (UFT1, UFT2 and UFT3) along with the species identified JCO1 and JCO2. In spite of that JCO1 is the same species that JCO5 (*T. asperelloides* GJS04-217) the JCO5 results were not significant. In the case of second screening method for 96 h of growth in CMC medium, the enzymatic halos presented values almost double in diameter compared with 48 h of growth. The highest values were of the strains JCO4, JCO2 and UFT2 with 71, 70 and 65 mm and with EI of 1.141, 0.971 and 1.283, respectively. However, the best enzymatic indices presented in this

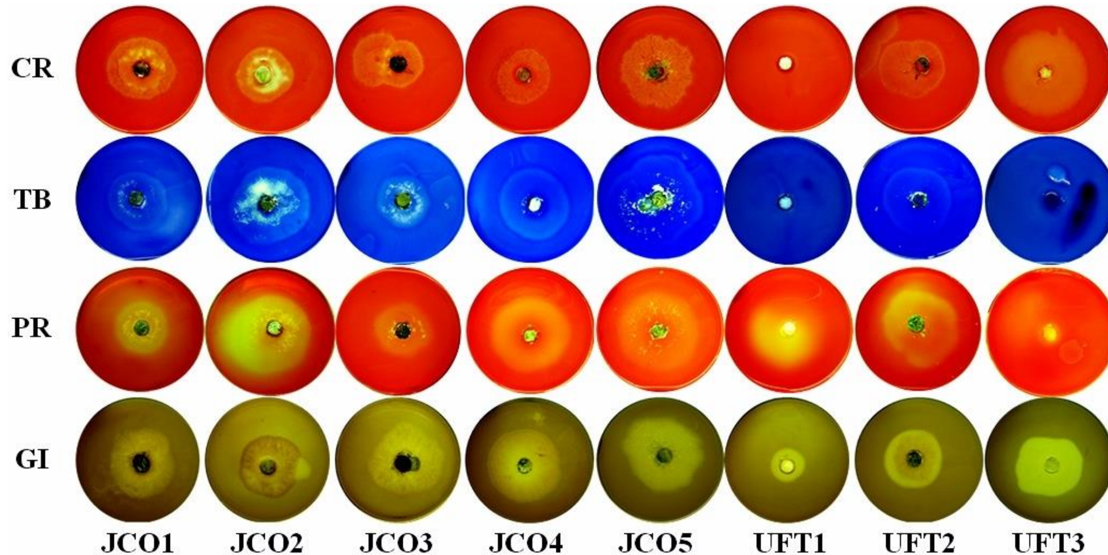

Figure 3. Evaluation of enzymatic activity in CMC medium (Carboxymethyl Cellulose 1% w/v) at 28ºC for 48 h using Congo Red (CR), Trypan Blue (TB), Phenol Red (PR) and Gram's Iodine (GI) as cellulase activity indicator.

method were evidenced by the strains UFT1, UFT3 and JCO1 with average values of 1.669, 1.440 and 1.373, respectively. Therefore, the previous results coincide with Tukey test; were, UFT1 and UFT3 belong to the group A and without significant differences between their values. Although, the Duncan test separate in different groups the strains UFT1, UFT3 and JCO1. Enzymatic indices data reported by Ruegger and Tauk-Tornisielo (2004) using the same screening method, where *Trichoderma harzianum* II presented EI of 1.0 and *Trichoderma longibrachiatum* did not provide growth of enzymatic halo, however, in the data found in this study, *T. longibrachiatum* DAOM 167674 (JCO2) and *T. harzianum* CIB T44 (JCO3 and JCO4) showed EI of 0.913, 1.272 and 1.185 of endo-glucanases respectively.

The screening performed in CMC medium (Figure 3) presented different color degradations. In Congo Red, the enzymatic halo change from red to opaque orange, the larger halos corresponded to strains UFT2, JCO5 and UFT3 with average values of 40, 39 and 38 mm, respectively. In Trypan Blue, the dark blue color change to light blue and the larger halos presented in UFT1, JCO2 and JCO5 with average diameters of 61, 57 and 56 mm, respectively. In Phenol Red, the red color was degraded by the enzyme production changing to yellow color, were the larger halos diameters were in the strains JCO5, JCO2 and UFT2 with average values of 57, 55 and 55 mm, respectively. In the case of Gram's Iodine, the medium presented a brown coloring initial and the enzyme secretion changed to beige, with higher average diameters in UFT3, JCO4 and JCO1 with values of 42, 38 and 37 mm. These strains despite having higher enzyme halos, the colony diameters were proportional. Therefore, were not necessarily the best to present a high enzymatic index.

The strains grown in Avicel medium (Figure 4) presented different colorations and enzymatic degradations compared with growths in CMC medium. In Congo Red, the red color changed to an orange color very similar to the original. The larger halos corresponded to strains JCO5, UFT2 and JCO4 with average diameters of 51, 48 and 46 mm, respectively. In Trypan Blue, the dark blue color changed to light blue color and the larger halos diameters in JCO5, JCO2 and UFT3 with average values of 45, 38 and 37 mm, respectively. In Phenol Red, JCO5, UFT3 and JCO4 presented the higher average diameters with values of 58, 49 and 48 mm, respectively. Were, the red color changed to yellow. Finally, the strains JCO5, UFT2 and JCO1 showed the best enzymatic diameters with average values of 52, 45 and 40 mm, respectively in Gram's Iodine dye. In the majority of the chromogenic indicators, the strain JCO5 presented the best enzymatic values. However, due to the proportional growth of the fungal colony, the best enzymatic indices corresponded to the strains UFT1, UFT3 and UFT2.

Conclusions

This study allowed us to identify the best strains for cellulases production such as Endo-glucanases and Exo-glucanases, the optimal growth conditions using two screening methods with two growth times and the chromogenic dyes most appropriated for growth halos measurement.

The eight fungal strains showed potential for production

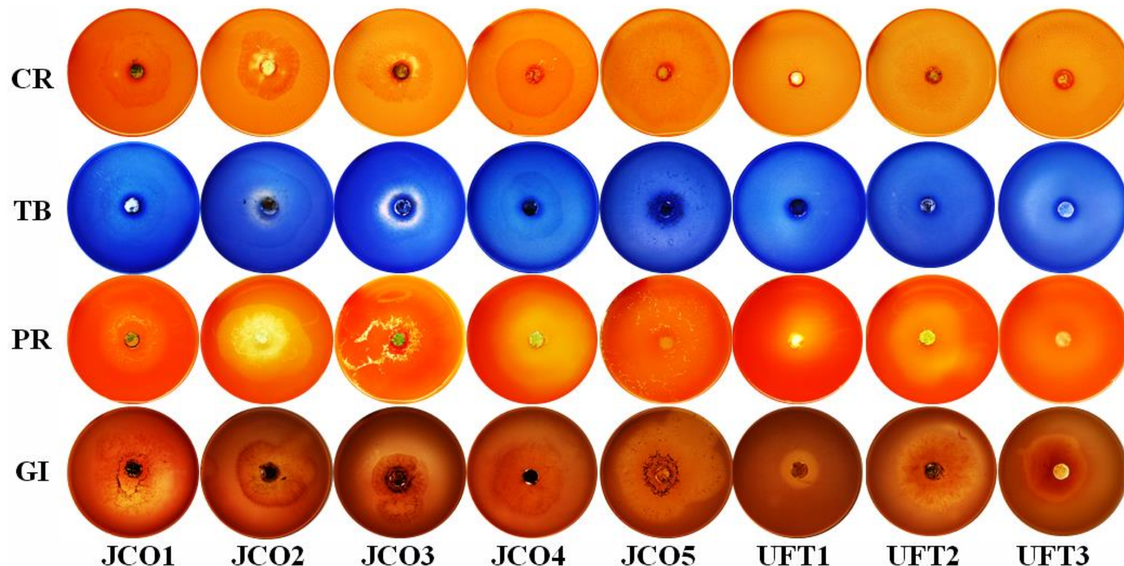

Figure 4. Evaluation of enzymatic activity in Avicel medium (Cellulose microcrystalline 1% w/v) at 28ºC for 48 h using Congo Red (CR), Trypan Blue (TB), Phenol Red (PR) and Gram's Iodine (GI) as cellulase activity indicator.

of cellulases. However, the best enzymatic indices of Endo-glucanases were presented by the strains UFT1, UFT2 and UFT3 with average values above 1.0. For the Exo-glucanases, the best indices were of the strains UFT1, UFT2 and UFT3 although the strains JCO1, JCO2 and JCO3 also had values above 1.0.

For screening methods, it was observed that the second screening method allowed verifying the results using double the time of growth enabling, the study of the production of enzymatic halos depending on the type of microorganism and its growth phase. However, the screening method for 48 h is ideal for rapid and effective detection of potential producers of cellulases.

In most cases, several researchers have used the dye Congo Red as chromogenic indicator for detection of cellulases (Jo et al., 2011; Sharma and Sumbali, 2014). However, in the present study, the best colorations were evidenced using Phenol Red in CMC medium with a best revelation of the contrast between the original color and the change caused by enzymatic degradation. Otherwise as occurred in Avicel medium, were the best views of contrast in the halos presented using Gram's Iodine also demonstrated by Kasana et al. (2008).

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Use of pectin in the postharvest conservation of tangerine

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The aim of the present study was to evaluate the postharvest behavior of tangerine coated with different pectin concentrations during storage under controlled temperature (22°C ± 0.1). Fruits with green color (± 90% of the surface) were divided into four groups: fruits without any coating (T1) and fruits coated with pectin solution at 4 g / 100 g (T2), 6 ml / 100 ml (T3) and 8 g / 100 ml (T4). Tangerines were evaluated during the storage period (0, 3, 6, 9, 12 and 15 days) for the following parameters: vitamin C, soluble solids (SS), total titratable acidity (TTA), mass loss, turgidity pressure and external appearance through colorimetric analysis. In general, coated fruits showed lower mass loss over the storage period. The polynomial model was the model that best suited the experimental data. Regarding to the physico-chemical characteristics, the citrus fruits and non-climacteric, showed little variation in the treatments and changes that have occurred and which can be explained by the variability of the fruits used. In general, the fruit treated with different concentrations of pectin kept green for longer period and with this feature of the fruits, is better accepted by the consumer.

Key words: *Citrus deliciosa* Tenore, pectin, coatings, coloring, mathematical models.

INTRODUCTION

Tangerine belonging to genus *Citrus deliciosa* Tenore is a citrus fruit originated from Asia. Trees that produce tangerines adapt to tropical and sub-tropical climates, have intermediate size and are thorny, with full and rounded crown formed by small leaves of dark green color.

It is considered a mid-season fruit, which is harvested primarily from May to June and due to seasonality, there is a need to extend its offer to other periods of the year (Nascimento et al., 2010). About 960 tons of tangerines are harvested annually in the country (IBGE, 2012). The quality characteristics of citrus fruit are of paramount

importance for marketing, whether for fresh consumption or for juice processing. Attributes that are of relevance in postharvest quality as include appearance, flavor, odor, texture and nutritional value. Chitarra and Chitarra (2005) report that postharvest fruit quality is related to the minimization of the respiration rate, firmness, color, appearance, aiming at keeping them attractive to consumer for a longer period of time.

According to Miguel et al. (2009), fruits of strong and bright coloring are preferred by consumers, although it is not a factor that contributes to their nutritional value. Many storage techniques have been developed over the years to increase the shelf life of fresh fruits, which can be extended by the use of controlled and modified atmosphere and use of edible coatings (Togrul and Arslan, 2004). Despite the wide availability of synthetic packaging and functionality, there is difficulty in recycling, and this has encouraged investigators to develop biodegradable materials with functional characteristics that allow its use as packaging (Souza et al., 2012).

Edible coatings are biofilms prepared from biological materials that act as barriers to external elements by protecting the packaged product from physical and biological damage and increasing its shelf life (Henrique et al., 2008). Coatings have excellent barrier properties, especially against the transport of gases and water vapor, and other factors that contribute to maintaining the postharvest quality of fruits. Edible films can help providing firmness and shine to coated fruits (Valencia-Chamorro et al., 2011).

Many studies with biofilms have been aimed at evaluating the physical and chemical quality of fruits and vegetables, among them: cassava starch in the conservation of sweet pepper; (Lemos et al., 2007) tomatoes coated with different pectin concentrations (Oliveira et al., 2012.); postharvest conservation of guavira coated with carboxy methyl cellulose, pectin and calcium chloride and pectin alone (Scalon et al., 2012.); tomatoes coated with FruitWax H2 carnauba wax, FruitWax M-AC emulsion resins and Meghwax carnauba wax (Chiumarelli and Ferreira, 2006); carnauba wax in the conservation of persimmon (Blum et al., 2008.); blackberry coated with cassava starch and water kefir grains (Oliveira et al., 2013). Despite the great diversity of biofilms used on fruits and vegetables, there is little information on the application of pectin-based biofilm on tangerines.

Pectin is of utmost importance in food technology and processing as it is associated with the function of providing firmness, flavor and aroma retention, as well to its role as hydrocolloid in the dispersion and stabilization of various emulsions (Gancz et al., 2006). Although, pectin extraction varies according to the raw material used, in general, the process comprises extraction of original vegetable in acid aqueous medium, purification of the liquid extracted and pectin isolation by precipitation (Christensen, 1984). The content of pectic substances varies according to the botanical origin of the plant material, four byproducts from agricultural and food industries rich in pectic substances (content above 15 g / 100 g dry basis): apple bagasse, citrus albedo, beet pulp and sunflower petals (Thibault, 1980). Above, the research aimed to evaluate the influence of coating the base of pectin in physical and chemical characteristics of mandarins (*C. deliciosa Tenore*) during storage.

MATERIALS AND METHODS

The study was conducted at the Laboratory of Fruits and Vegetables - Food Engineering sector, Federal Institute of Goiás - Rio Verde Campus, and the colorimetric evaluation was performed at the Food Engineering sector, School of Agronomy, Federal University of Goiás (Goiânia).

Materials

The fruits were harvested at its full physiological development (± 90% of the surface), being obtained by manual harvesting in a farm in the region of Rio Verde - GO (17° 37'38.26 "S; 50° 45'18.94"W; Altitude: 704 m). Fruits were washed in running water, sanitized with sodium hypochlorite solution at 100 $mg.kg^{-1}$ solution for 15 min and dried with paper towel. Fruits were then randomly divided into four groups: tangerine with no coating, composing the control treatment (T1) and fruits coated with pectin solution at 4 g / 100 ml (T2), 6 g / 100 ml (T3) and 8 g / 100 ml (T4).

Preparation and addition of coatings

Preparation of proposals for concentrations of the biofilm, we used the following amounts of commercial citrus pectin maker Dinâmica - Química Contemporrânea Ltda (formulation 1 L): 40 g (solution at 4 g / 100 ml); 60 g (solution at 6 g / 100 ml) and 80 g (solution at 8 g / 100 ml). Formulations were homogenized in a semi-industrial blender (Skymsen 15V-04) for 30 s until pectin gelation. After preparation, tangerines were immersed in different solutions (± 1 min) and placed in metal racks to dry naturally. Subsequently, they were placed on polystyrene trays for BOD storage with controlled temperature (22 ± 0.1°C) for 0, 3, 6, 9, 12 and 15 days.

Physical and chemical analyses

Every three days of storage, whole fruits were submitted to turgidity pressure, by flattening technique using a horizontal leveler (Calbo and Nery, 1995) and to determine the mass loss of tangerines in each treatment three replicates were formed, and each repetition contained five fruits. The weight of the fruits was performed on an analytical balance CELTC FA 2104M, with results expressed in grams. We used the same fruits to evaluate the evolution of the mass loss during storage (AOAC, 2005), and after manual pulping,

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Table 1. Mathematical model applied for mass loss.

Model	Model designation
Page	$PM = \exp(-k \cdot t^n)$
Linear	$PM = k \cdot t$
Modified Page	$PM = \exp((k \cdot t)^n)$
Logarithm	$PM = a \cdot \exp(-k \cdot t) + c$
Handerson and Pabis	$PM = a \cdot \exp(-k \cdot t)$
Two Terms	$PM = a \cdot \exp(k_0 \cdot t) + b \cdot \exp(k_1 \cdot t)$
Polynomial	$PM = k_0 + k_1 \cdot t + k_2 \cdot t^2 + k_3 \cdot t^3$

t: Storage time, days; k, k_o, k_1, k_2, k_3: model constants, $days^{-1}$; and a, b, c, n: model coefficients.

the following chemical analyses were performed: the vitamin C content was determined according to the methodology described by Lutz Institute Adolf (1985) by titration with potassium iodide and the results expressed as mg vitamin C / 100 mL of juice, soluble solids based on direct reading refractometer Atago N-2E (ITB, 2005), the results being expressed in brix and the total acidity according to the methodology described by AOAC No. 942.15 (1997), by titration with NaOH 0.1 M was determined, and the results expressed as g total acid / 100 mL. For mass loss, fruits were numerically marked and weighed during storage.

Color

Tangerines were evaluated in terms of instrumental color parameters according to the CIELab system L *, a * and b * in colorimeter (ColorQuest II, Hunter Associates Laboratory Inc, Virginia), where three distinct points were analyzed, totaling 24 points in each fruit. The results were expressed in L *, a * and b * values, where L * values (luminosity or brightness) ranged from black (0) to white (100), a * values from green (-60) to red (60), and b * values from blue (-60) to yellow (60).

Mass loss

From the experimental data obtained by the weight loss, they were adjusted to different equations shown in Table 1 commonly used to represent the rate of mass loss of agricultural products during storage. The use of different equations for mass loss study is relevant to have a higher precision.

Statistical analyses

Experiments were conducted in a completely randomized design (CRD) in 4 x 6 factorial scheme consisting of four treatments (control, 4, 6 and 8 g / 100 ml pectin) and six storage times (0, 3, 6, 9, 12 and 15 days) using software R (2014), where the analyzes were conducted in three replicates for each treatment, and each replicate consisted of 5 fruits, and each analysis was performed in triplicate for each repetition. The results were submitted to analysis of variance by F test and the comparison of means by the Tukey test at 5% probability. For statistical analysis of mass loss, mathematical models were fitted by nonlinear regression using the Gauss-Newton method and statistical software. The models were selected considering the magnitude of the determination coefficient (R^2), Chi-square test ($\chi 2$) and standard deviation of the estimate (SE).

$$SE = \sqrt{\frac{\sum\left(Y - \hat{Y}\right)^2}{GLR}}$$

$$\chi^2 = \frac{\sum\left(Y - \hat{Y}\right)^2}{GLR}$$

Where, Y: value experimentally observed; $\hat{Y}$: value estimated by the model; GLR: degrees of freedom (number of experimental observations minus the number of model coefficients).

The criteria used for selecting the model were the magnitude of the determination coefficient (R^2), the lowest standard deviation of the estimate (SE) and the $\chi 2$ value (chi-square).

RESULTS AND DISCUSSION

Chemical characterization

During storage, acids present in fruits are used as substrates for respiration, reducing their content. The titratable acidity values (Figure 1) showed small variations ($p \leq 0.05$) as a function of treatments and storage time. In general, the vitamin C content (Figure 2) decreased as a function of the storage time all treatments. The applied coatings were used in an attempt to prevent the transfer of gases between atmosphere and fruit. Accordingly, coatings should help prevent the oxidation of vitamin C by preventing fruit exposure to oxygen and changing enzyme activity. However, samples showed statistically equal losses, with little or no variation. Azeredo et al. (2012) reported that acerola fruit coated with mixtures of nano-reinforced polymeric materials showed lower levels of ascorbic acid when compared to a single coating. Alleoni et al. (2006) also observed a slight change in TS and SS values in juice from orange coated with films based on milk whey protein concentrate. Arnon et al. (2014) reported that the TS and SS levels in juice are important parameters of internal quality in citrus and did not observe any significant effects

Table 2. Mean determination coefficient (R^2), standard deviation of the estimate and χ2 values for mathematical models of mass loss for coated tangerines.

Model	R^2 (%)	SE (decimal)	χ^2
T1			
Page	98.94	1.20	1.45
Linear	98.93	1.08	1.17
Modified Page	98.94	1.20	1.45
Logarithm	99.15	1.25	1.55
Handerson & Pabis	90.31	3.64	13.28
Two Terms	90.31	5.15	26.56
Polynomial	99.63	1.01	1.02
T2			
Page	98.61	0.96	0.93
Linear	96.01	1.46	2.12
Modified Page	98.61	0.96	0.93
Logarithm	97.42	1.51	2.29
Handerson & Pabis	87.05	2.93	8.61
Two Terms	92.85	3.08	9.51
Polynomial	99.62	0.71	0.50
T3			
Page	98.68	0.97	0.94
Linear	98.88	0.80	0.64
Modified Page	98.68	0.97	0.94
Logarithm	99.73	0.51	0.26
Handerson & Pabis	94.33	2.00	4.01
Two Terms	97.88	1.73	3.00
Polynomial	99.76	0.58	0.34
T4			
Page	97.98	1.27	1.62
Linear	96.70	1.46	2.12
Modified Page	97.98	1.27	1.62
Logarithm	99.15	0.96	0.92
Handerson and Pabis	94.59	2.08	4.35
Two Terms	94.59	2.95	8.69
Polynomial	99.41	0.98	0.95

either using commercial waxes or coating mixtures on the TS and SS levels of juices from any of the varieties tested in the study.

The SS content of control and coated fruits showed an increasing trend, which may be related to the mass loss of fruits. The behavior of soluble solids observed in this study corroborates with the results obtained by Nascimento et al. (2011) and Obenland et al. (2011).

Turgidity pressure and mass loss evaluation

Firmness is a critical quality attribute for consumer acceptability. Coated fruits showed significant turgor values compared to control fruits during the study period. Pectin coatings had a beneficial effect on fruit firmness so that at the end of the storage period, all treatments led to higher fruit firmness values compared to control fruits ($p = 0.05$) (Table 2).

In general, coated fruits showed higher firmness values over the storage time, and treatments T3 and T4 were the most efficient; however, at the end of the storage period, treatment T2 showed peel stiffness, which is not related to turgidity pressure, but rather to a physiological disorder by CO_2 accumulation promoted by atmosphere modification, water loss and high variability of fruits used.

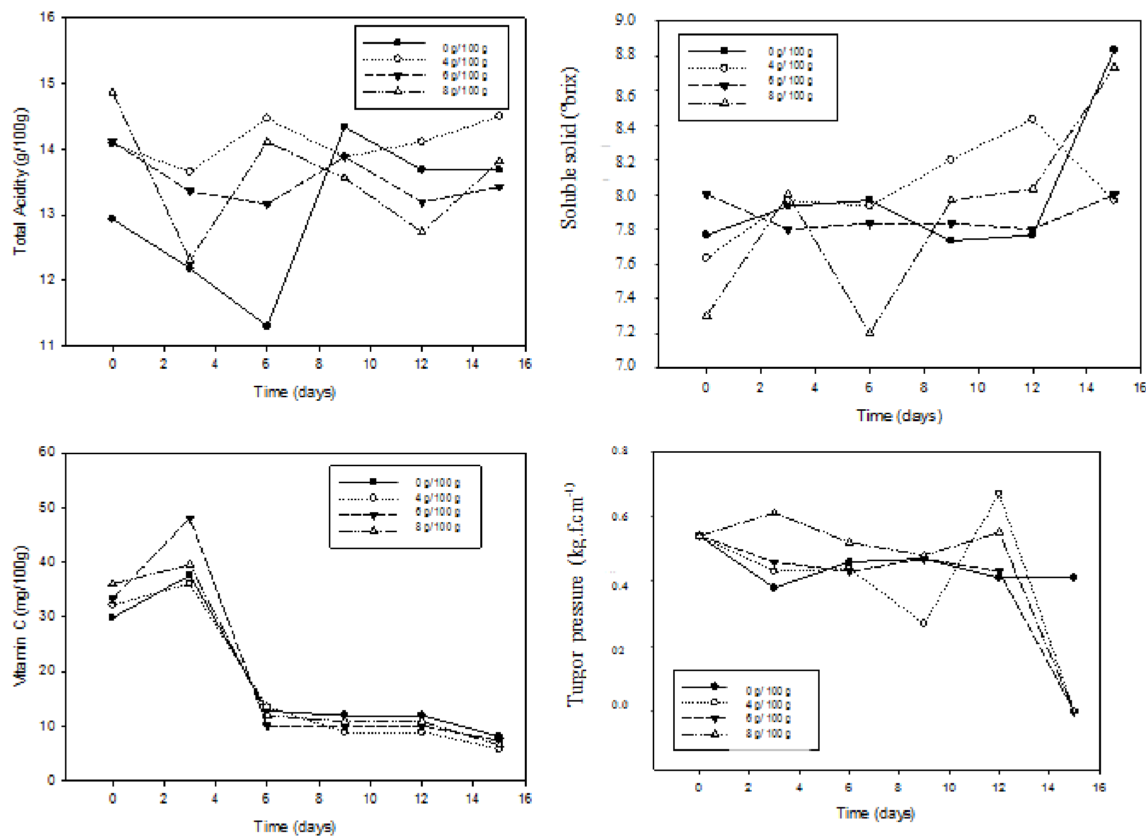

Figure 1. Titratable acidity (g/100 g), solubles solids, vitamin C and turgor pressure of tangerine coated with pectin -based biofilm stored at 22°C.

Arnon et al. (2014) reported on studies with different citrus that the modification of the internal atmosphere of fruits causes CO_2 accumulation and can stimulate anaerobic respiration. Table 2 shows the R^2, χ2 and SE values. By analyzing the determination coefficient, it turns out that the polynomial model obtained the highest values for all treatments and Two Terms and Henderson and Pabis models showed the lowest values. According to Doymaz (2012), the determination coefficient is one of the main criteria for the selection of the model that best fits the drying process; however, in addition to R^2, parameters SE and χ^2 are used to determine the fitness quality. Analyzing the average deviation of the estimate (SE), all models showed low values; however, the polynomial model ($PM = k_0 + k_1 \cdot t + k_2 \cdot t^2 + k_3 \cdot t^3$) showed the lowest values for all treatments. According to Draper and Smith (1981), the ability of a model to adequately represent a given physical process is inversely proportional to the mean deviation of the estimate. Table 2 also showed that through the chi-square test, it appears that only Two Terms and Henderson and Pabis models for the control treatment (T1) showed calculated chi-square values higher than tabulated values ($\chi^2_{tabulated}$ = 11.070), which discards the use of these models for the representation of this phenomenon. The polynomial model showed the lowest chi-square values for all treatments. Günhan et al. (2005) point out that the lower the chi-square value, the better the model fits the study phenomenon.

According to the criteria adopted, the polynomial model was chosen to represent the mass loss process of stored coated fruits because this model was the one that best suited to all treatments. Togrul and Aslan (2004) worked with tangerines coated with carboxy methyl cellulose and concluded that the polynomial model was the most suitable to represent the fruit mass loss. Figure 3 shows the mass loss *versus* storage time of fruits coated with different pectin concentrations adjusted by the polynomial model. The mass loss analysis allowed verifying the effectiveness of coatings in delaying the loss of water from fruits. The beneficial effects of fruit coatings included improved appearance and reduced mass loss. Machado et al. (2012) reported that there is a highly significant interaction between coatings and shelf life of fruits, reducing their mass loss. Shi et al. (2013) evaluated the effect of coatings on postharvest longan fruits and concluded that coatings significantly reduced the mass loss of fruits. Mathematical models describe mass loss as a function of storage time. Experimental data are closely linked to data estimated by the polynomial model, which

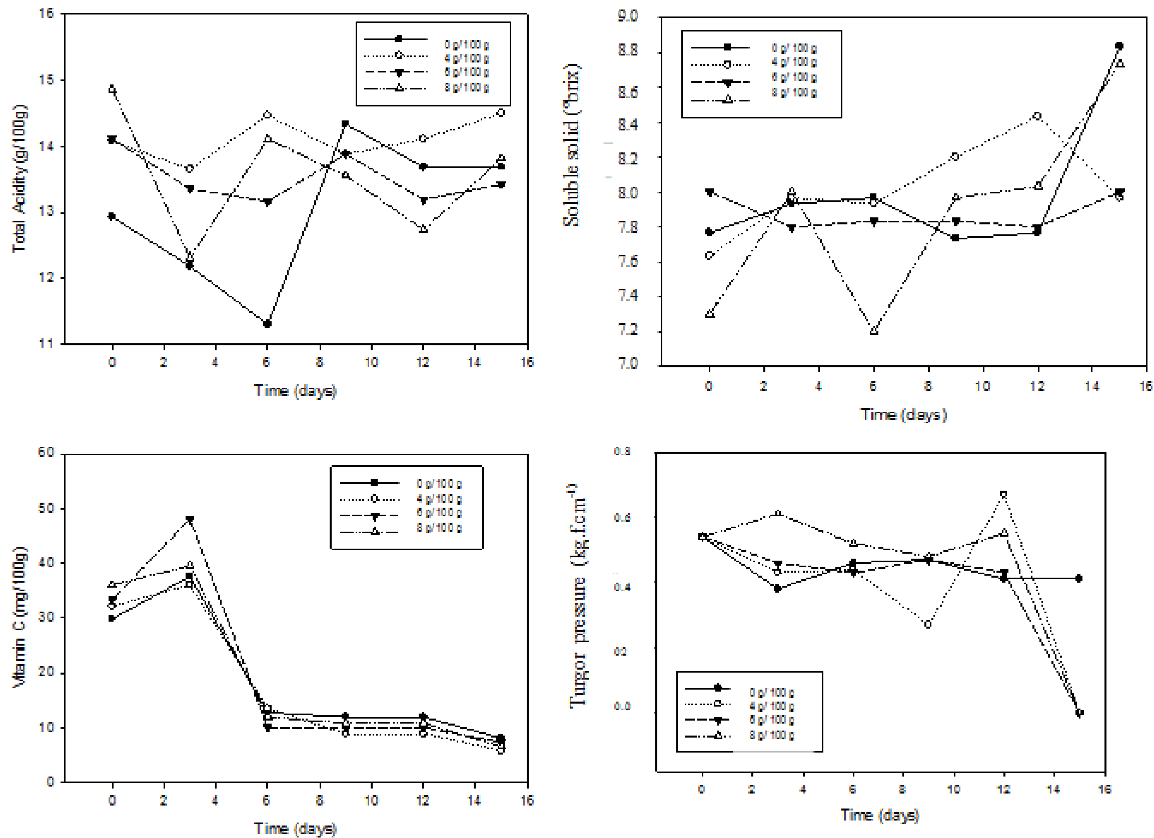

Figure 2. Titratable acidity (g/100 g), solubles solids, vitamin C and turgor pressure of tangerine coated with pectin - based biofilm stored at 22°C.

Table 3. Polynomial model fitted to experimental data of mass loss during storage.

Treatment	Polynomial model
T1	$PM = - 0.2350 + 2.6209t - 0.0922t^2 + 0.0029t^3$
T2	$PM = - 0.1780 + 2.4570t + 0.8916t^2 + 0.4155t^3$
T3	$PM = - 0.1359 + 0.8916t + 0.0473t^2 - 0.0012t^3$
T4	$PM = - 0.0505 + 0.4155t + 0.1183t^2 - 0.0036t^3$

indicates the suitability of the model to describe the mass loss of fruits with and without coating. Table 3 presents the polynomial equations with constants obtained for the mass loss process. Constant "k" is related to water loss rate during storage processes and coatings, as barriers against water loss, influence the "k" values. By analyzing data, it was observed that constant "k" decreases in absolute values with increasing pectin concentrations in fruits, a fact expected, since higher coating concentrations lead to less transfer of water to the environment.

Colorimetric analysis

Luminosity or L * represents the brightness of the fruit surface. Increase in L * value is related to fruit ripening, and coatings, as barriers to gas exchange, slow biochemical reactions of chlorophyll degradation, reducing ripening and therefore increasing the L * values. Significant difference (p = 0.05) between treatments was observed for all parameters.

By analyzing the luminosity values of fruits during the storage period (Figure 3), it was observed that pectin coatings influenced gradually with concentrations used. Fruits coated with 8 g / 100 mL of pectin showed smaller L * values, a fact related to chlorophyll preservation. Importantly, fruits with more attractive features are better marketed. Asgar et al. (2010) worked with tomatoes coated with gum Arabic and concluded that fruits kept luminosity values stable during storage. Asgar et al. (2011) reported that papaya fruits coated with higher chitosan concentrations obtained slower changes in

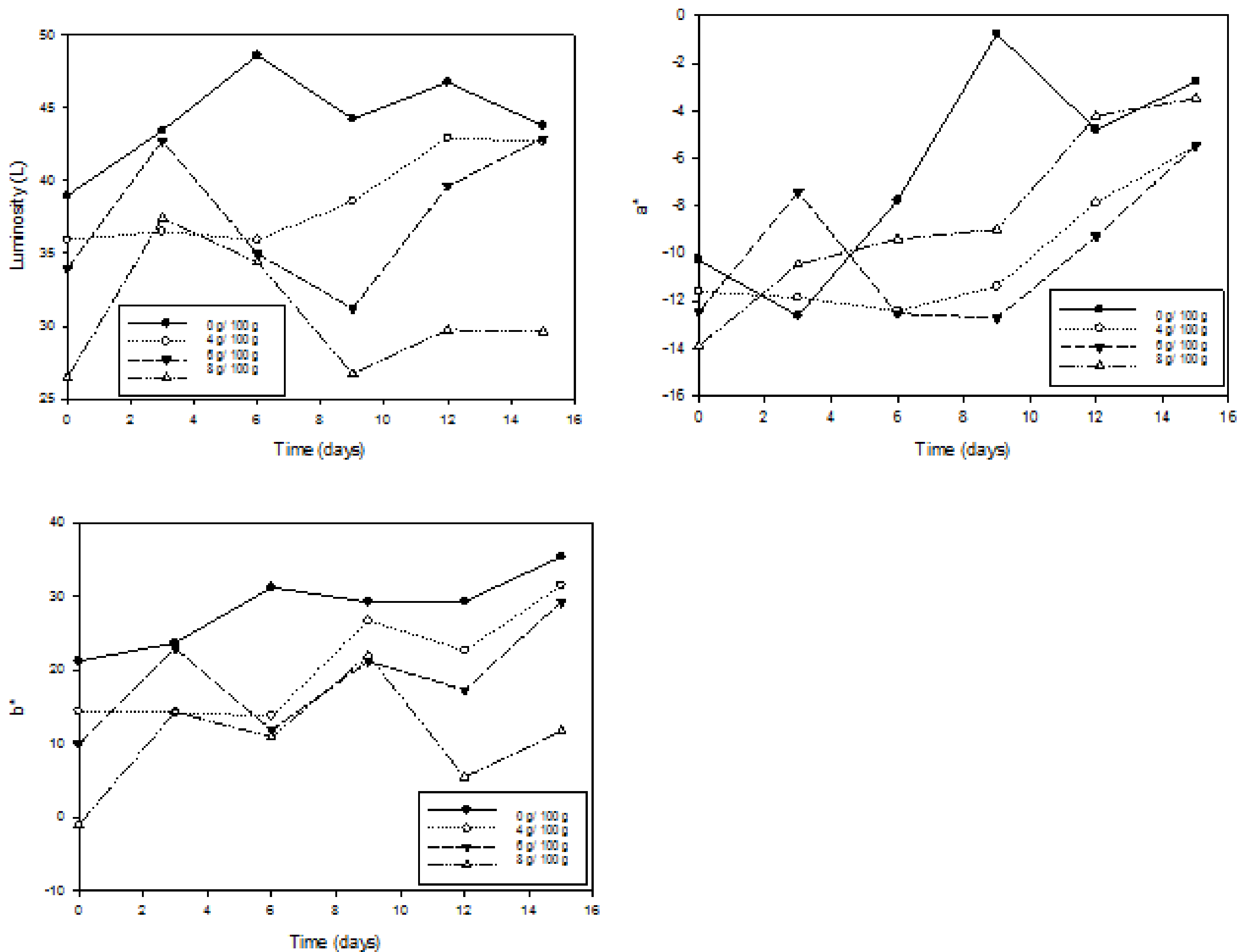

Figure 3. Luminosity (L *), green intensity (-a *) and yellow intensity (+ b *) of tangerine peels coated with pectin-based biofilm stored at room temperature.

the L * variable, corroborating the present work. Rojas-Grau et al. (2009) observed that chitosan-based coatings decreased the browning of pears during storage because the biofilm forms a barrier against gas exchange. In general, pectin-based treatments influence variable a * up to the fifteenth day, being therefore effective to keep the green color of fruits. During ripening, tangerine peel goes from green to yellow, which is related to chlorophyll degradation and revelation of carotenoids (Jacomino et al., 2008). Alvares et al. (2003) found a variation of coordinate -a *, which defines the green color in bananas (Ribeiro, 2006). The yellow intensity (+ b *) of fruit peel presented in Figure 3 showed great variance throughout the storage period for Control fruits and for treatments T2 and T3, and revealed a significant increase in the yellow color of fruits. The advancement in the b * values is the sign of maturation of fruits, with great influence for marketing. For fruits with higher pectin concentration (8 g / 100 mL), the advancement in b * values was less expressive and it could be inferred that the pectin concentration was enough to prevent the "yellowing" of fruits. Chroma is the relationship between a * and b * values, in which the real color of the object being analyzed is obtained. The results of the chroma parameter corroborate the b * results, in which control and treated fruits had 4 g / 100 ml and 6 g / 100 mL pectin, with significant values for yellow color and fruits with 8 g / 100 mL pectin, keeping color green. Statistically, at the beginning of the storage period, treatments can be considered equal, with variability occurring from the third day. The statistical difference for fruits with 8 g / 100 mL pectin over time is much less pronounced when compared to other treatments, and it could be concluded that fruits maintained green color over the storage period. Maftoonazad and Ramaswamy (2005) studied postharvest avocados and reported that

coated fruits showed a slower rate of change in peel color. Ali et al. (2010) reported that coated tomatoes were greener even after 20 days of storage compared to control.

Conclusion

Mathematical models are suited to fruit mass loss, being useful tools to describe the mass loss during storage. The polynomial model best fitted to experimental data. The use of pectin-based coatings reduced the fruit mass loss. Fruits coated with 8 g / 100 ml pectin can be stored for 15 days at 22°C. The results presented confirm the beneficial effect of pectin films in postharvest conversation of tangerine fruits. Coatings are a simple, environmentally friendly and relatively inexpensive way to extend the shelf life of tangerine fruits.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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