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Full Length Research Paper

Response of maize (*Zea mays* L.) to combined application of organic and inorganic (soil and foliar applied) fertilizers

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A field trial was carried out in the 2013 cropping season at the Teaching and Research Farm of Kwara State University, Malete, Nigeria (08° 42" 48.5N and 004° 26 17.9" E) to assess the response of early maturing maize variety (TZEE-Y) when using organic poultry manure (pm) alone or in combination with inorganic (NPK) and foliar fertilizer (ff) (boost xtra). The treatments were: pm 5.0 t ha^{-1} + ff, pm 2.5 t ha^{-1} + NPK 30 kg N + ff, NPK 60 kg N + ff, pm 5.0 t ha^{-1} + NPK 60 kg N, pm 10.0 t ha^{-1} + 60 kg N, NPK 120 kg N/ha, pm 10.0 t ha^{-1} and control. The treatments were arranged in a randomized complete block design and replicated three times. Applications of poultry manure at 10.0t ha^{-1} produced the highest plant height (119.57 cm^2), leaf area (362.10 cm^2) and cob length (17.47 cm^2). However, significantly, was at par with integrated application of poultry manure at 2.5 t ha-^{1} mixed with NPK 30 kgN ha^{-1} and foliar fertilizers. As compared to other treatments, significantly shorter days (38) to 50% flowering was obtained where 2.5 t ha^{-1} poultry manure was combined with NPK 30 kgN/ha and foliar fertilizer. The highest grain yield (3.206 t ha^{-1}) was obtained when pm was applied alone at 10.0 t ha^{-1}. This was also similar to the combined application of pm at 2.5t ha^{-1} mixed with NPK 30 kgN ha^{-1} and foliar fertilizer. The results of the study indicated that combined application of pm, NPK and ff enhanced the growth and yield of maize. This integrated application will be a good soil management practice for tropical soils. Combination of pm at 2.5 t ha^{-1} with NPK 30 kgN ha^{-1} and foliar fertilizer (boost xtra) is therefore recommended for early maturing maize production in the study area.

Key words: Poultry manure, foliar fertilizer, mixing, NPK fertilizer, application rate.

INTRODUCTION

Maize is an important cereal crop in Nigeria, mainly as an energy giving food with a total production of 7.3 million tons (FAO, 2007). Being a versatile crop, constituting about fifty percent in the poultry feed ingredients, it is

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widely cultivated in all the agro-ecological zones in Nigeria. The diverse use of maize as food for man and his livestock and raw materials for industries has made the crop in continuous production. Compared to other arable crops such as millet, the nutrients requirement of maize is quite higher and hence, constituting major constraints to its production.

Application of inorganic fertilizer to increase crop growth and yield is well known since the nutrients are readily available for plant use but continuous and inappropriate use of inorganic fertilizer is harmful both to the soil and the environment. It increases soil acidity, and nutrient imbalance and pollution of underground water. In view of the well documented detrimental effects of inorganic fertilizer, its rising cost and unavailability has limited its use among poor farmers in Nigeria (Taminu et al., 2007), hence, attention has been directed to the use of organic manure in recent times.

Historically, poultry manure (pm) has long being recognized as a source of enriching plant and amendments of soil nutrient. It contains all the essential nutrients including the micronutrients such as cupper, manganese and zinc and has been reported as a valuable source of plant nutrients (Garg and Bahla, 2008) and also improved the physical, chemical and biological properties of the soil (Abou El-Magd et al., 2006). Combination of pm with inorganic soil applied fertilizer has been extensively used on various crops to improve growth and yield. Mixing the two sources of fertilizer not only supply essential and micro nutrients for plant use, but also can have some positive interaction to increase their efficiency thereby reducing environmental hazards particularly soil pH (Bayu et al., 2006). Makinde and Ayoola (2001) observed that combined application of organic and inorganic fertilizer increased the yield of maize (*Zea mays* L.) than when any of the fertilizer was used alone. Similarly, Akande et al. (2003) reported significant improvement on the growth and yield of okra (*Abelmuschus esculenthus* Moench) when ground phosphorous rock phosphate was mixed with poultry manure. In a recent study on sweet maize (*Z. mays* L. var saccharata Strut), Uwah et al. (2011) reported that application of poultry manure at 10 t ha^{-1} mixed with 400 kg ha^{-1} NPK fertilizer out-yielded other treatments in biomass yield, harvest index and total grain yield.

The practice of applying liquid fertilizer to plant leaves (foliar fertilization), is recently done in Nigeria, and it is gradually gaining popularity among peasant farmers in many cultivated crops. This method of fertilizer application has been reported to increase the growth, yield and quality of crops such as okra (Selvi and Rani, 2000), soybean (Barge, 2001) and tomato (Alexander et al., 2004) among others. Philips (2004) demonstrated that this technique apart from supplying the micronutrients it also acts as a catalyst in the uptake and use of certain macronutrients. Boost xtra, is a foliar fertilizers that is commonly used by farmers in Nigeria. It is manufactured by Candel Company and contain both the macro and micro nutrients in various combinations (20% N, P and K, 0.075% Zn, Cu and Mg, 1.5% Fe, 0.35% Mn, 0.035% Bo and 0.012% Mo with pH range of 4.0-4.5). Many studies had been carried out on the integration of pm and inorganic soil applied fertilizers, but limited information is available on the use of pm and inorganic fertilizer with foliar fertilizer (ff) in this particular area. Therefore, the objective of our study is to evaluate the growth and yield of an early maturing maize variety (TZEE-Y) with the combined application of organic pm, NPK and ff.

MATERIALS AND METHODS

A field trial was carried out in 2013 cropping season at the Teaching and Research Farm of Kwara State University, Malete, (08° 42" 48.5N and 004° 26 17.9"ı E) in the Southern Guinea Savannah ecological zone of Nigeria to evaluate the response of an early maturing maize (TZEE-Y) variety to the combined application of inorganic NPK (15:15:15), organic pm and inorganic foliar fertilizers (ff). The mean annual rainfall of the study area during the trial was 900 mm in 54 rainy days. The maximum temperature was 35.5°C while minimum was 22.8°C with relative humidity of 85.9% (PME, 2013). The treatment consists of poultry manure at 10 t ha^{-1}, NPK at 120 kg N ha^{-1}, pm at 5.0 t ha^{-1} plus ff, pm at 2.5 t ha^{-1} + NPK at 30 kg N/ha + ff, NPK at 60 kg N/ha + ff, pm at 5.0 t ha^{-1} + NPK at 60 kg N ha^{-1}, pm at 10 t ha^{-1} + NPK at 60 kgN ha^{-1} and the control where none of the fertilizers was applied. The treatments were arranged in a randomized complete block design and replicated three times.

Soil samples were collected at 0-15 cm depth at the experimental site before planting for laboratory analysis. After the experiment, that is, after harvesting of the crops, soil samples were also collected from each plot for laboratory analysis (Table 1). The land was ploughed and harrowed twice and planting was carried out on the flat at a spacing of 0.75 m between rows and 0.50 m within the rows. Each plot size measured 3 m x 3 m with 0.5 m between the plots and 1 m between the blocks. Poultry manure was applied two weeks before planting while NPK was split applied at three and six weeks after planting (wap). Foliar fertilizer was applied in a single application at the recommended rate of 3 l ha^{-1} at tasselling. Pendimethalin [N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine] was applied as a pre-emergence herbicide at the rate of 1.5 kg a.i ha^{-1} immediately after planting and followed by manual weeding at four weeks after planting. The following data were collected from five tagged plants at the two inner rows; plant height at 6 wap with a measuring tape from the ground level to the base of the last leaf, leaf area, stem girth using Vernier caliper, days to 50% tasselling and silking. Maize cobs from the two inner rows were harvested and the yield and yield components data were taken viz: cob length, number of cobs $plant^{-1}$, weight of 1000 grain and grain yield. The data were further subjected to analysis of variance (ANOVA) using Assistat Statistical package (2009) version and treatment means were separated using Duncan's multiple range test at 5% level of probability.

RESULTS

The physiochemical properties of the soil at the experimental site before planting and after harvesting the crop are presented in Table 1. The soil of the experimental site was predominantly sandy with above 88.9%

Table 1. Physico- and chemical properties of the soil at the experimental site before planting and after harvesting.

Treatment	pH in H_20 (1:1)	N (%)	OC (%)	OM (%)	Sand (%)	Silt (%)	Clay (%)	P	Ca	Mg	Na	K
Before planting	5.8	0.40	0.43	0.74	88.9	6.0	5.04	1.40	2.4	1.1	0.74	1.53
After harvesting												
pm 10 t + NPK 60 N/ha	6.0	0.21	0.39	0.67	80.9	12.0	7.04	3.50	2.2	0.5	0.95	2.00
pm 10 t/ha	6.7	0.18	0.32	0.55	84.9	10.0	5.04	2.90	1.9	0.6	0.86	2.46
pm 5 t + ff	6.0	0.14	0.29	0.50	84.9	8.0	7.04	2.80	1.3	1.3	1.13	1.89
pm 2.5 t + NPK 30 N + ff	6.3	0.14	0.37	0.64	84.9	8.0	7.04	3.50	0.3	0.3	1.08	1.85
pm 5 t + NPK 60 N/ha	6.4	0.15	0.28	0.48	84.9	8.0	7.04	3.5	0.8	0.8	1.26	2.05
NPK 60 kg N/ha + ff	6.5	0.11	0.36	0.62	80.9	12.0	7.04	2.80	0.5	0.5	0.74	1.74
NPK 120 kg N/ha	6.7	0.14	0.24	0.41	82.9	10.0	7.04	0.70	0.8	0.8	0.78	2.15
No fertilizer	6.0	0.11	0.19	0.33	84.9	8.0	7.04	0.70	0.7	0.7	1.21	1.64

OC, organic carbon; OM, organic manure.

Table 2. Effect of combined application of poultry manure, NPK and foliar fertilizer on plant height, stem girth, leaf area and days to 50% tasseling of maize.

Treatment	Plant height (cm)	Stem girth (cm)	Leaf area (cm^2)	Days to 50% tasseling
pm10 t/ha	119.57a	7.47a	362.1a	43.15b
pm10 t/ha + NPK 60 N/ha	116.90a	7.57a	331.3bc	40.67c
pm 5 t/ha + NPK 60 N/ha	74.27f	7.10a	348.7b	43.07b
pm 2.5 t/ha + NPK 30 + ff	115.27ab	7.20a	353.07a	38.03d
pm 5 t/ha + ff	100.10cd	7.60a	308.4de	46.78a
NPK 60 + ff	83.37e	6.23b	295.97e	46.00a
NPK 120 kg N/ha	110.20b	6.23b	329.60bc	44.2b
Control	46.9h	5.80b	248.47f	47.11a

Values with the same letter in the column are not statistically different at 5% level of probability using Duncan multiple range test. Pm = poultry manure; ff = foliar fertilizer.

sand, 6.0% silt and 5.04% clay, slightly acidic and low in some macro and micro nutrients. Integrated application of pm and inorganic fertilizers significantly influenced the growth of maize (Table 2). The greatest plant height (199.57 cm) and leaf area were recorded at the treatments where pm was applied alone at 10 t ha^{-1}. Comparable plant heights were obtained with the integrated application of pm at 2.5 t ha^{-1} mixed with NPK 30 kg N ha^{-1} plus ff and the combined application of pm at 5.0 t ha^{-1} and foliar fertilizer. Significantly, shorter plants were observed at the control treatments as compared to other treatments. Stem girth followed similar trends with the plant height, but the application of pm alone at 10 t ha^{-1} was only

Table 3. Effect of combined application of poultry manure, NPK and foliar fertilizer on cob length, number of cobs per plant, weight of 1000 grains and yield.

Treatments	Cob length (cm)	Number of Cobs per plant	Weight of 1000 grains (g)	Yield (t/ha)
pm 10 t	17.47a	1.33ab	32.50a	3.206a
pm 10 t + NPK 60 N/ha	17.0ab	1.53a	30.87b	3.170a
pm 5 t + NPK 60 N/ha	14.70d	1.13ab	25.27e	2.559b
pm 2.5 t + NPK 30 + ff	16.97ab	1.20ab	30.76b	3.1567a
pm 5t + ff	16.17bc	1.40ab	29.27c	2.502b
NPK 60 + ff	14.60d	1.20ab	29.10c	2.106bc
NPK 120	16.18b	1.20ab	30.43b	2.90ab
Control	11.83e	1.00b	27.33d	1.29d

Values with the same letter in the column are not statistically different at 5% level of probability using Duncan multiple range test Pm = poultry manure; ff = foliar fertilizer.

only superior to the control. Integrated application of pm (10 t ha^{-1} + NPK 60 kg N ha^{-1}), pm (5 t ha^{-1} + NPK 60 kg N ha^{-1}) and pm (2.5 t ha^{-1} + NPK 30 kg N ha^{-1} + ff) produced similar leaf areas. Plots treated with combined application of pm at 2.5 t ha^{-1} mixed with NPK 30 kg N ha^{-1} and foliar fertilizer commenced tasselling earlier at 38 days after planting. Late tasselling was recorded at the control treatments.

The combined integration of pm, NPK and foliar ff on the cob length, 1000 grain weight, number of cobs per plant and yield of maize is presented in Table 3. Application of pm alone at 10 t ha^{-1} was superior in cob length as compared to other treatments except when it was combined with NPK 60 kg N ha^{-1}. Foliar fertilizer mixed with pm at 5.0 t ha^{-1} produced similar cob length with when NPK was applied alone at 120 kg N ha^{-1}. The numbers of cobs produced per plant in all the treatments were statistically alike. The highest grain weight (32.50 g) was recorded when pm was applied alone at 10 t ha^{-1}. Mixing pm (2.5 t ha^{-1} + NPK 30 kg N ha^{-1} + ff) and pm (10.0 t ha^{-1} + NPK 60 kg N ha^{-1}) had similar 1000 grain weight. These values were however similar to when NPK was applied alone at 120 kg N ha^{-1}. Applications of pm alone at 10 t ha^{-1} or in combination with NPK 60 kg N ha^{-1} and at a reduced rate of 2.5 t ha^{-1} mix with NPK 30 kg N ha^{-1} plus foliar fertilizer statistically out-yielded other treatments. Minimum grain yield (1.29 t ha^{-1}) was observed at the control treatments.

DISCUSSION

The low essential plant nutrient content of the soil at the experimental site indicated the need for external soil amendment for sustainable yield to increase maize yield in the study area. The inherent low nutrient soil condition at the experimental site was reported by Adejobi and Kormawa (2002) which could be due to negative nutrient imbalance that is often associated with intensive cropping and inappropriate application of inorganic fertilizer in the traditional cropping in the tropics. Generally, all the treatments improved the textural properties of the soil (sand, silt and clay), the available phosphorous and the soil pH. This further confirmed the earlier findings of Akande et al. (2003, 2010) that application of organic materials could ameliorate slightly acidic tropical soils to improve crop production. The overall results of the study indicated that application of pm alone or in combination with inorganic NPK and ff improved the growth, yield and yield components of maize. These findings were consistent with the findings of other researchers (Khaliq et al., 2004; Uwah et al., 2011).

Application of pm at high rate of 10 t ha^{-1} though improved the growth and yield, similar values were recorded when it was mixed at a reduced rate of 2.5 t ha^{-1} with NPK 30 kg N ha^{-1} plus foliar fertilizer. This combination also compared favorably with the recommended NPK 120 kg N ha^{-1} in all the observed parameters. This clearly suggests that the recommended high dose of 10 t ha^{-1} pm and 120 kg N ha^{-1} could be reduced to one quarter when mixed with foliar fertilizer to achieve reasonable maize yield. This reduced rate however, contradicted the earlier recommendation of Akande et al. (2010) that one half each of pm and NPK is ideal for maize yield. The contrast is explainable with the mixture of ff that was not included in the earlier study.

Nutrient availability and use for maize appeared to be better with the combination of the three sources of fertilizers. The slow release of nutrients from pm was complemented with the application of NPK and ff as evident in the early tasselling in the plots treated with the combined application of the three sources. Application of plant nutrients to the leaves where chemical processes of photosynthesis takes place is the quickest way of nutrient utilization by plant. This is because the nutrients are delivered at the site where they are quickly used by the plant. This method of fertilizer application in addition to the replenishment of micronutrients also acts as a catalyst in the uptake and use of macronutrients (Phillips, 2004). Earlier, Boateng et al. (2006) recommended the

combined application of pm and NPK on the growth and yield of maize because of its complementary and synergistic effects. These positive attribute appeared to be further strengthened with the application of ff.

Based on the results of this study, combined application of pm, NPK and ff enhanced the growth and yield of maize. Mixing ff with pm and NPK will reduce the high dosage of each of the fertilizers required per unit area, improve soil properties and could be a sound soil management strategy for sustainable maize production in the tropics. Hence, 2.5 t ha^{-1} PM plus 30 kg N ha^{-1} and full rate of foliar fertilizer is therefore recommended in the study area.

Conclusion

Combined application of pm, inorganic and ff enhance the growth and yield of maize. However, application of 2.5 t ha^{-1} of pm plus 30 kg N ha^{-1} and full rate of ff (boost xtra) was found to give similar yield with recommended pm and inorganic N fertilizer.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Susceptibility of Algerian pepper cultivars (*Capsicum annuum* L) to *Phytophthora capsici* strains from different geographic areas

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Ten pepper cultivars (*Capsicum annuum* L), that is, Doux D'Alger, Sonar, Esterel, Doux, Marconi, Magister, Belconi, Italico II, Lipari, Arabal, and Doux d'Espagne commercially grown in Algeria were inoculated with six isolates of *Phytophthora capsici* Leon, and susceptibility of pepper was assessed by evaluation of disease severity in different plant organs. The cultivar Italico II was found to be more resistant to *P. capsici* isolates compared to other cultivars, for example Esterel which was the very susceptible one. In addition, the pepper cultivars differed significantly ($P < 0.01$) in their susceptibility to different *P. capsici* isolates, on the rate of stem necrosis. However, isolated virulent fungal isolates displays similar pattern when they were inoculated to the studied cultivars ($P > 0.05$). However, velocity of disease development varied between resistant pepper cultivars, that is, Italico II and susceptible ones, for example Esterel. In conclusion, data suggest that pepper cultivars differ in their susceptibility to *P. capsici* isolates.

Key words: Susceptibility, *Capsicum annuum*, *Phytophthora capsici*, greenhouse conditions.

INTRODUCTION

Disease caused by *Phytophthora capsici* strains, is one of the serious issues for peppers grown in Algeria and in other regions of the world (Silvar et al., 2006). It was first identified in the Mesilla Valley of southern New Mexico in 1922 and is considered the causal agent of pepper wilt and infects virtually any under-ground or upper-ground organs of pepper plants (Ristaino and Johnston, 1999; Hausbeck and Lamour, 2004; Tamietti and Valentino, 2001). The disease is manifested by the appearance of several symptoms with various alteration forms of all plant organs (Manohara, 2007). Pathogen translocation and consequently disease outbreak is largely due to latent infection in plantlets and the contaminated nursery medium (Aravind et al., 2011).

Due to the high susceptibility of pepper cultivars to *P. capsici*, pesticide treatments are used before and after crop settlement in the field to control this pathogen (Hwang and Kim, 1995). However, chemical control has various environmental and safety limitations and often sometimes is ineffective against *Phytophthora* strains.

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Many studies have indicated that the biological control approach is not effective (Oelke et al., 2003). The effectiveness of chemical control of pepper root rot, on the other hand, is only minimal, hence the efforts to identify resistant/less susceptible pepper cultivars. Similarly, several fungicides against this disease are available, but their effectiveness varies with respect to experimental conditions. The fact that *P. capsici* is a soil pathogen makes natural and chemical control very difficult (Thabuis et al., 2003). The development of adapted *Phytophthora*-resistant pepper cultivars is considered to be an essential approach for the control of *Phytophthora*. Hence, it is necessary to screen for varieties/ cultivars resistant or tolerant to *P. capsici* that persist in soil (Divya and Sharada, 2014).

The aim of this investigation was to understand the susceptibility of commercially cultivated sweet pepper cultivars, in three regions of Algeria to *P. capsici* isolates.

MATERIALS AND METHODS

Plant material and growth conditions

Ten sweet pepper (*Capsicum annuum* L) cultivars were used in this study. They are recognized in Algeria as Doux D'Alger, Sonar, Esterel, Doux Marconi, Magister, Belconi, ItalicoII, Lipari, Arabal, and Doux d'Espagne. Most varieties were hybrids except Doux d'Espagne, Doux Marconi, and Doux d'Alger which are stable (ITCMI, 2001). They were sown under greenhouse conditions in plastic conical pots (4^2 × 6.5 cm) containing sterile black peat and sand (2/3 and 1/3 v/v). Each pot was seeded with one or two seeds. Cultures were irrigated until soil saturation and regularly every 3 days, and they were incubated at 20 to 25°C for 45 days (Biles et al., 1992). Seedlings were transplanted to plastic conical pots (12^2 × 21 cm) containing steam sterilized mixture of sand and loam soil. Seedlings were irrigated with a mineral solution for 45 days. Small seedlings were collected and washed well with tap water and sterile distilled water, and then they were placed in glass bottles containing 150 ml of NPK solution (15:15:14 w/v) and 50 ml of Richard medium. The pH of the mixture was 7.8.

Sampling, isolation and identification of *P. capsici*

Isolates of *P. capsici* were collected from symptomatic plant tissues, including roots, stems, and fruits of sweet pepper. Sweet pepper samples were obtained from three different areas in Algeria, that is, Jijel, Constantine and Biskra, known as important pepper producing regions. They were transported to the laboratory and stored overnight under refrigeration until analysis. Plant tissues were surface-disinfested with 95% ethanol, and a small piece from lesion margins was placed on V-8 juice agar. The dishes were incubated at 25°C in the dark for 7 to 10 days. Six strains of *P. capsici* were selected for further studies. Strain J1 from spotted leaves and strain J2 from rotten roots were obtained from Jijel region; strain C1 from rotten fruit and strain C2 from rotten roots were isolated from Constantine region; whereas, strain B1 from rotten roots and strain B2 from diseased stems were isolated from Biskra region. Thereafter, isolates were observed under a microscope, and were identified based on morphological characteristics (Tsao and Alizadeh, 1988; Gerrettson-Cornell, 1989; Stamps et al., 1990; Tsao, 1991). Cultures of *P. capsici* were regenerated monthly on V-8 juice agar and conserved at 22°C.

Artificial inoculation of pepper plants

Stems, leaves and roots of pepper plants (*C. annuum* L.) of different varieties were artificially contaminated with *P. capsici* Leon isolates. The experiments were conducted in a controlled room at temperature 22 ± 2°C, 12 h photoperiod and 100% relative humidity. Young fungal strains (less than 10 days of age) were used, and pepper plants were in the vegetative phase (Barksdale et al., 1984). Pathogenicity of the six isolated fungal strains and resistance limits of different organs of pepper cultivars were studied. The score of the virulence of *P. capsici* was evaluated by measuring the necrotic spots on stems, leaves and roots.

Inoculation of stems

Seedlings of the ten pepper cultivars were adapted to the new environment for 5 days before the start of the experiment. Then, stems were decapitated under the last leaf, and a disc (4 mm in diameter) was punched out from a young culture of each fungal isolate cultivated on a solid medium. Each disc was placed on injury created on the stem of each pepper cultivar. The applied inoculums were covered with an aluminum foil covering the entire cross section of each stem as a chamber to maintain high humidity at the top of the stem. Necrosis extension was measured every 3 days for 15 days. 180 plants (pots) are used, the 10 tested cultivars are inoculated by the 6 isolates of fungus, thus 3 replications were done, and this test was done 2 times. The measured daily necrosis rate provides useful information about the rate of mold development in the stem tissue (Pochard et al., 1976).

Inoculation of leaves

Mature pepper leaves of different cultivars (Doux d'Alger, ItalicoII, and Esterel) were used to score resistance levels, when inoculated by a highly virulent strain of *P. capsici* Leon (Isolate J2). Leaves were placed in Petri dishes on a thin film formed by sterile distilled water. They were inoculated in the middle veins with a mycelial disc (Ø =4 mm in diameter). The tested leaves were 18 inoculated with one isolate (J2) each cultivar is represented by 6 leaves, each two leaves are put in a Petri dish, that experience was repeated twice. The resistance of leaves was estimated by measuring the necrotic spots extension from the inoculation site after 24, 48, 72 and 96 h (Molot et al., 1984).

Inoculation of roots

Sweet pepper cultivars (Lipari, Esterel, ItalicoII and Belconi) were used for studying resistance levels of roots. Before transplanting plants into glass bottles containing mineral solution, 4 discs (Ø= 4 mm) of a newly growing virulent fungal strain on V-8 juice agar were thrown into this liquid to release motile zoospores. The tested plants were inoculated with one isolate (J2), each cultivar is represented by 10 plants, this test is repeated 3 times, and the total number of plants (pots) tested is 120. Plants death may have resulted from direct effects of root rot or crown rot. Mortality percent was used to estimate pepper resistance (Satour and Butler, 1967; Yildiz and Delen, 1979).

Statistical analysis

Data were analyzed by the one way analysis of variance (ANOVA) and the test with $P<0.05$ was considered as statistically significant. This was followed by Fisher's test when the number of treatments was under than 5 and over 2 (leaf and root treatments), and Duncan's

Table 1. Length* of necrosis caused by *Phytophthora capsici* Leon isolates on stems of different sweet pepper cultivars.

Pepper cultivar	*Phytophthora capsici* Leon isolates						Mean necrosis to all isolates for every cultivar
	J1	J2	C1	C2	B1	B2	
Doux D'Espagne	53	69	59	68	42,66	31	53,77666667ih
Doux Marconi	56	69.66	58	66.33	40	34.33	54,05333333h
Belconi	98.33	112.33	93.66	109.66	91	85	98,33ed
ItalicoII	48.66	61.66	33	60.66	25.33	19.66	41,495i
Sonar	113.66	129.66	106.66	124.33	100.33	97.66	112,05b
Doux d'Alger	98.33	113.66	85	111.66	87	85.66	96,885gef
Esterel	122.33	139	118	134.66	113	105.66	122,1083333a
Magister	106.33	120.66	104.66	113.66	100.33	97.33	107,1616667cb
Lipari	99.33	123.33	85.33	120	83.33	81.33	98,775
Arabal	100	119.66	98.66	119.33	93	89	103,275dc
Mean necrosis in all cultivars For every isolate	89,597	105,862	84,197	102,829	77,598	72,663	

J1……B2: isolates of *Phytophthora capsici* L (J1 and J2: from Jijel, C1 and C2: from Constantine and B1 and B2: from Biskra). *length of necrosis is measured in mm. The same lower case letter in table cells sign that there are no significant differences between cultivars as Sonar (b) and Magister (cb), the two cultivars have the same b. Cells contain the different letters appear that there are significant differences like Esterel (a) and Italico II(j) or Doux d'Alger (gef)and Doux d'Espagne (ih).

Duncan's when the number of treatments were over 5(stem treatments).

RESULTS

Fugal isolation and characterization

Colony and sporangium morphology were variable. The colony shapes observed were stellate, rosaceous, and radial. Sporangium shapes observed were ellipsoid, globose, obovoid, ovoid, and distorted. The fungus did not form chlamydospores. The growth rate of all isolates was similar at 25°C. The overall optimum temperature for ve--getative growth was between 25 and 30°C. Some isolates grew optimally at 25°C, while others grew best at 30°C.

Evaluation of the resistance in stems

Results of incidence and severity of stem necrosis caused by different *P. capsici* Leon isolates against ten sweet pepper cultivars is shown in Table 1. In general, most of pepper plant cultivars were highly susceptible to the studied fungal isolates. Brown necrosis on inoculated stems was observed for all plants. Mean necrosis length ranged from 80 to 140 mm for most pepper plants analyzed (7 cultivars). The other three cultivars (Doux d'Espagne, Doux Marconi and Italico II) developed less length necrosis on their stems (< 70 mm). The results indicate that Italico II was highly resistant cultivar and Esterel, in contrast was the most sensitive to the highest virulence fungal isolates selected from roots J2 (Figure 1) and C2. In addition, statistical analysis (Two way ANOVA) indicates that, pepper cultivar have significant effects on the rate of stem necrosis ($P < 0.01$; ddf: 85/80; $F = 5.124$) and the necrotic lesion length ($P < 0.01$; ddf: 9/80, $F = 482.899$) caused by different *P. capsici* isolates (Figure 1). In contrast, fungal isolates displays similar pattern when they were inoculated to the studied cultivars ($P > 0.05$). Italico II cultivar was the most resistant. Comparing, the disease severity and velocity using the highly virulent fungal isolate (J2), significant differences were obtained between stem necrosis development velocities of the resistant pepper cultivar Italico II and the susceptible cultivar Esterel (Figure 2). Considering the most sensitive cultivar (Esterel), stem necrosis length increased linearly and a constant development velocity (9.26 mm/day) during the evolution of infection and suddenly stopped. In contrast, the most resistant pepper cultivar (Italico II), stem necrosis developed with irregular velocity. Necrosis increased in length from the third day to the sixth one post-treatment and became slower during the next experimental period. Fungal isolate, as a playing factor, affects very significantly pepper resistance pattern ($P < 0.01$; ddf: 5/80, $F = 4148.054$). Figure 3 presents virulence levels of selected six *P. capsici* Leon isolates. The results indicate clearly that the isolate J2 (from the Jijel region) was the highly virulent one, while isolate B2 (from Biskra region) has low virulence potential.

Disease development on detached leaves

Inoculation of detached leaves, btained from three

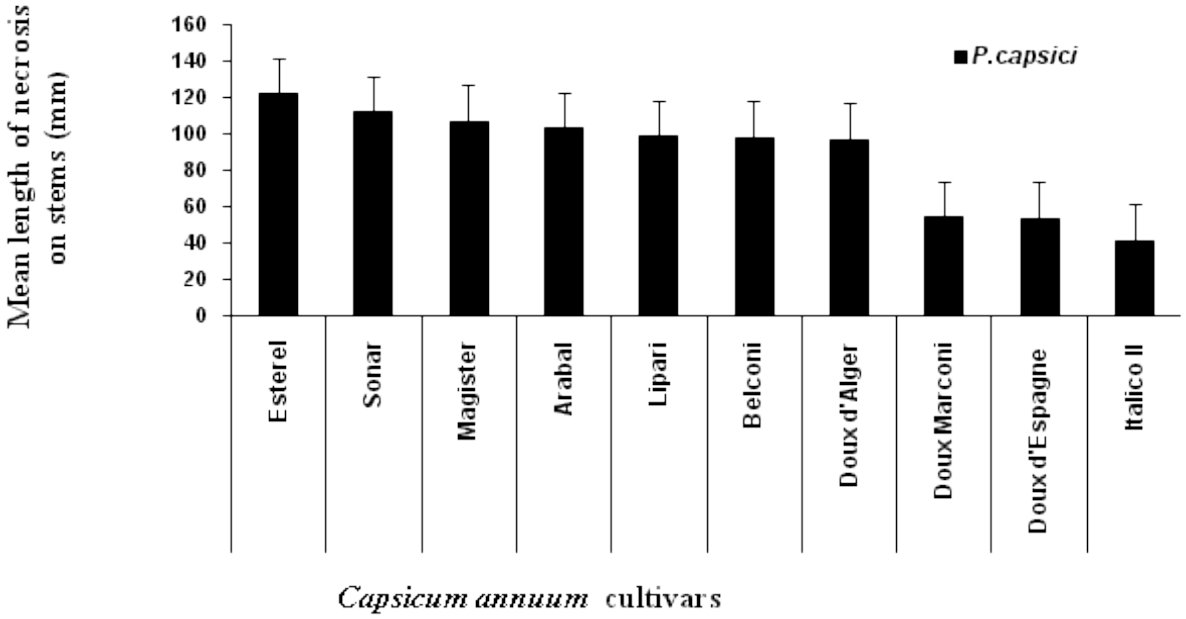

Figure 1. Resistance measurement of pepper cultivars (*C. annuum* L) according to stem necrosis length due to *P. capsici* Leon isolates.

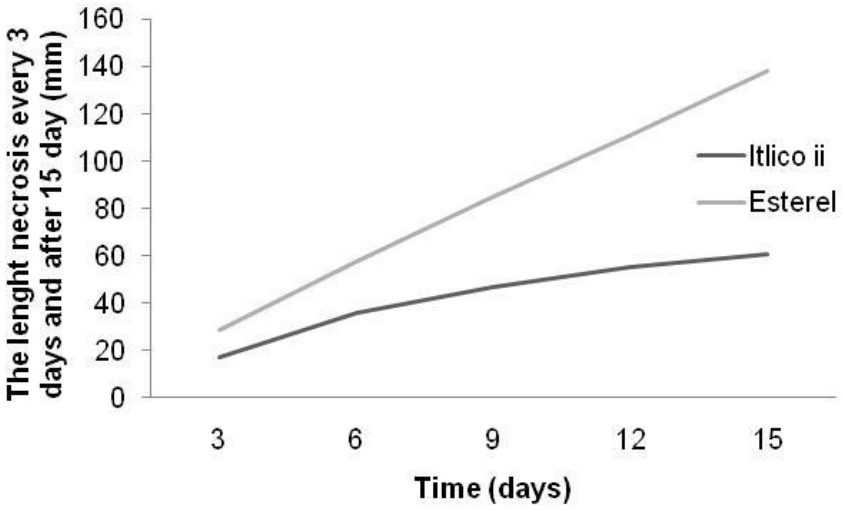

Figure 2. Stems necrosis development velocity due to *P. capsici* Leon (J2) in resistant (Italico II) and susceptible (Esterel) pepper (*C. annuum* L) cultivars.

different pepper cultivars, with the virulent fungal isolate (*P. capsici* J2) allowed the evaluation of the pepper plants pattern resistance (Figure 4). At the beginning of the experiment, wet dark green necrotic spots were developed, and then turned out to brown colour. Statistical analysis revealed clearly significant differences ($P < 0.01$; ddf: 2/15, F = 525,441) between disease symptoms in ItalicoII, Esterel and Doux d'Alger leaves pepper cultivars during 96 h post-inoculation. In the first cultivar, limited necrotic spots with average diameter 5.5 mm appear. In contrast, in the case of the two other susceptible cultivars, that is, A and B, larger necrotic spots with average diameter of 22.33 mm and18.41, respectively, were expressed (Figure 4). Leaf spot necrosis development in the three studied cultivars followed two different rates. Similar pattern, with constant development rate was observed in both Esterel and Douxd'Alger cultivars (5.6 and 4.6 mm/ day respectively) during the course of

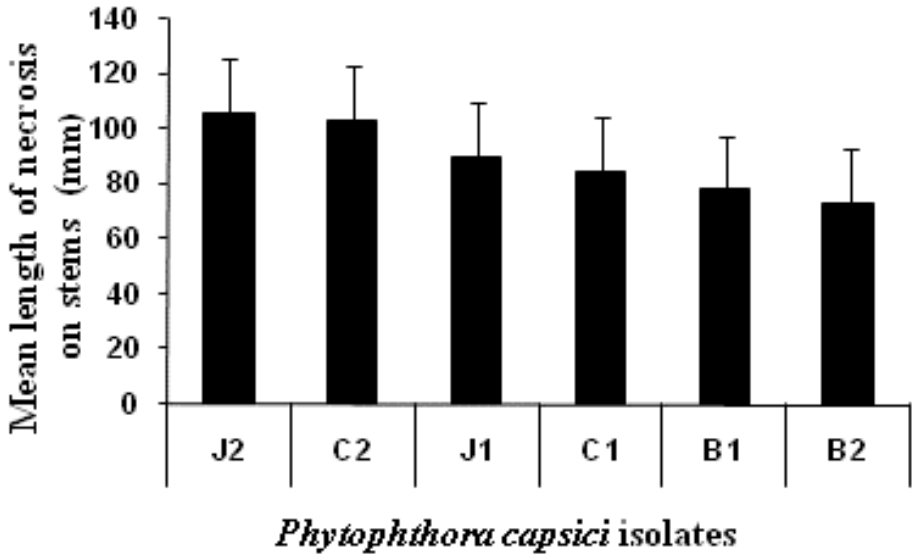

Figure 3. Virulence levels of *P. capsici* L. isolates on stem length necrosis appeared on pepper (*C. annuum* L) varieties. J1……B2: isolates of *Phytophthora capsici* L (J1 and J2: from Jijel, C1 and C2: from Constantine and B1 and B2: from Biskra).

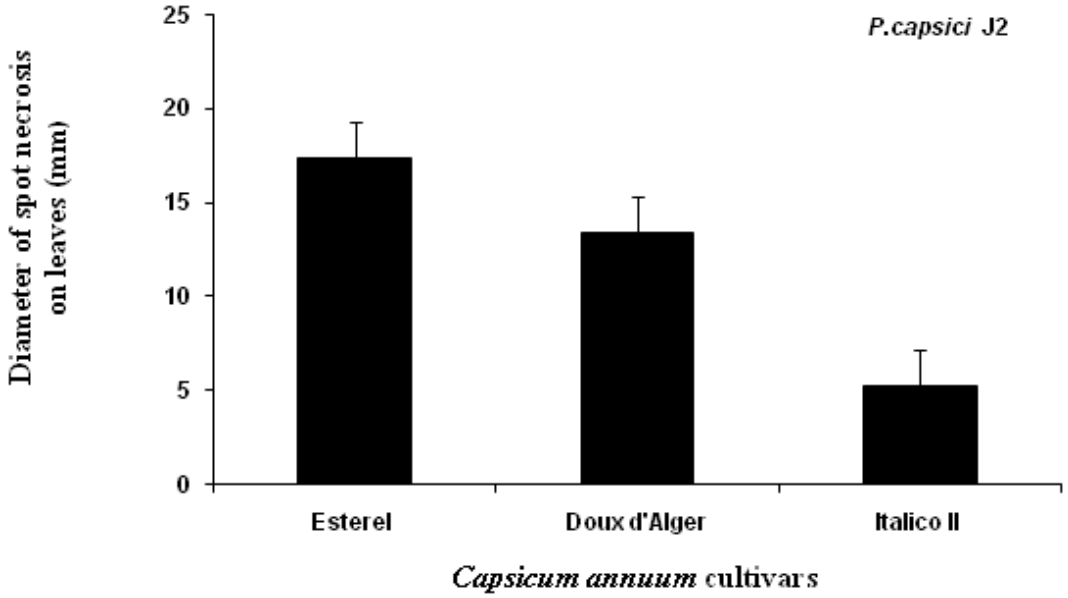

Figure 4. Spot necrosis diameter on leaves of three sweet pepper cultivars (*C. annuum* L, ItalicoII, Esterel and Douxd'Alger) expressed after 96 h post-inoculated with *P. capsici* Leon (J2).

infection. However, diameter of leaf spot necrosis in Italico II cultivar increased with a slower rate (1.4 mm/day) during the four days post-inoculation. Therefore, it seems that Italico II was the most resistant cultivar, compared to the other ones, that is, Esterel and Douxd'Alger. Italico II cultivar expresses the resistant pattern in the third day (Figure 5).

Evaluation of resistance in roots

Inoculation of four cultivated pepper cultivars (Lipari, Esterel, Belconi and Italico II) with the virulent *P. capsici* Leon isolate "J2" allowed the evaluation of the pepper plants susceptibility to this pathogen by measuring the plant mortality. Infection and brown-grey necrosis of

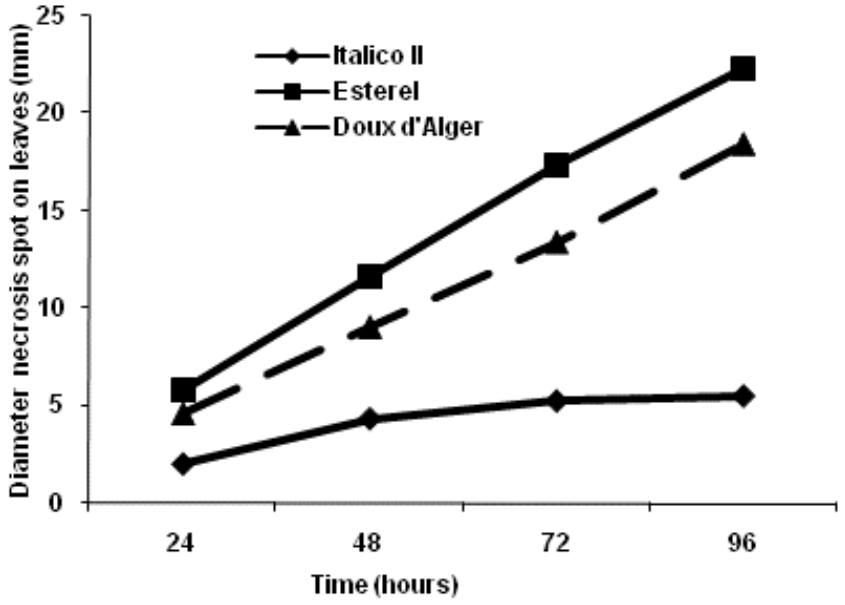

Figure 5. Spot leaf necrosis development rate (mm/day) expressed on detached sweet pepper leaves inoculated with a virulent isolate of *P. capsici* Leon (J2) for four days.

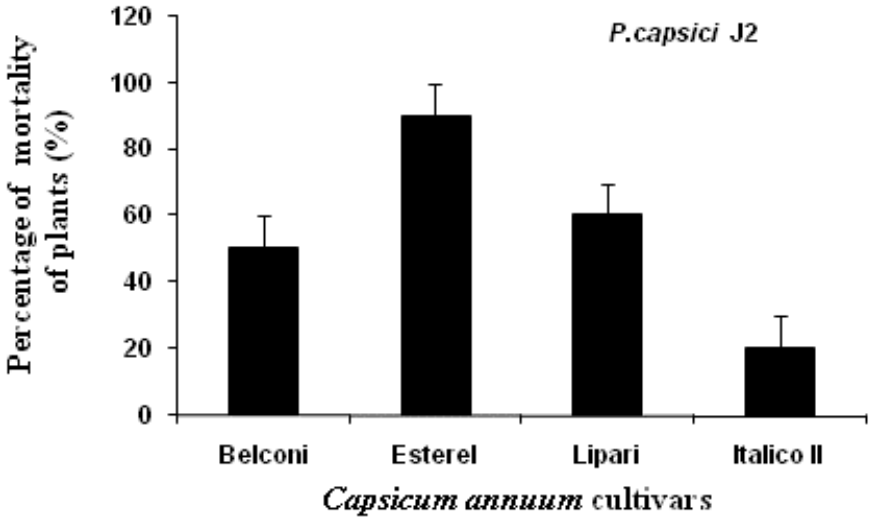

Figure 6. Percentage mortality among four cultivated sweet pepper cultivars (*C. annuum* L) inoculated with virulent *P. capsici* Leon (J2).

various plant parts were observed (root, root collars, plant tissues and plant crown). The infected plants wilt, dray and die consequently. Depending on cultivar type, significant differences between percentage mortality of cultivated plants were recorded ($P < 0.01$; ddf: 3/8, f = 21, 443). It seems that Italico II was the most resistant cultivar with only 16.66% mortality. In contrast Esterel, was the highly susceptible cultivar with 70% mortality. In addition, moderate resistance 43.33 and 40% mortality rate was recorded in the case of "Lipari" and "Belconi" cultivars, respectively (Figure 6).

DISCUSSION

Artificial inoculation of different organs (stems, leaves, and roots) of pepper (*C. annuum*L) with a range of strains of *P. capsici* Leon, led to the appearance of symptoms

similar to those described previously (Clerjeau et al., 1976; Kim and Hwang, 1992; Walker and Bosland, 1999; Foster and Hausbeck, 2010; Koç and Üstün, 2012.). Considering necrosis on pepper stem, velocity of disease development varied between resistant pepper cultivars (Italico II) and susceptible ones (Esterel). Furthermore, highly significant cultivar/isolate effects were found, indicating a differential host-pathogen interaction. The ability of a virulent fungal strain to induce necrotic lesions on stems depends on several factors especially plant defence mechanisms. Foster and Hausbeck (2010) reported a difference between pepper cultivars to different *P. capsici* strains after an artificial inoculation conducted in greenhouse. The same results were also obtained by Walker and Bosland (1999), Andrés Ares et al. (2005), Byron et al. (2010) and Koç and Üstün (2012) studying the interaction of pepper/ *P. capsici* isolates.

According to study of Clerjeau et al. (1976), the application of 23 isolates of the fungus *P. capsici* Leon on two varieties resistant "Phyo" and susceptible "YoloWonder" of pepper (*C. annuum* L) varying significantly, have given answers between the two varieties. Both isolate 13 and isolate 101 of the fungus gave necrotic lesions significantly shorter on the resistant variety compared to the isolates 96 and 112, and the opposite was observed when they were applied to the susceptible variety. The heterogeneity of genetic material plants is the explanation of heterogeneity of resistance responses (Pochard et al., 1976). There were other examples of strain-range interaction, where more reference strains were used. More aggressive, strains 71, 73 and 107 are less discerning between the two varieties. In any case, the criterion is the total length of necrosis extended for a week or 10 days, most of this period the stems of the variety "Yolo Wonder" may have a whole necrosis in the presence of more aggressive strains (Clerjeau et al., 1976). Studying the total length of necrosis on the stem, they found that the speed of development has a relationship with the development of the mycelial hyphae (Molot et al., 1976). They found that after a period of rapid decline in the rate of necrosis, in partner resistant (R), it is stabilized by a constant value and varies depending on the strain used, that characterizes the behavior of our range "Italico II", but in partner susceptible (S), the speed begins to increase after stabilizes with a high value that will decrease rapidly, stability is less permanent, we take into consideration that the fungus is rapidly approaching the base of the stem, and the best representative of this behavior is the cultivar "Esterel" (Pochard and Daubèze, 1980). The expression of resistance to the various organs of pepper is quantitative, is different depending on the variety. The genes responsible for this natural resistance reduce the rate of penetration of the fungus *P. capsici* Leon in pepper tissues (Pochard et al., 1983). The resistance is expressed discontinuously between the organs in some varieties or continuously in others, such as the case of the varieties tested, the resistance remains at the root level associated with a high level of resistance in the stem. According to Pochard and Daubèze (1980), the sensitivity of the roots to the fungus *P. capsici* Leon may be linked to partial loses of resistance from the summit to the crown of the plant,

In addition, the fact that there was a great variation in virulence among fungal isolates would reflect the possible occurrence of pathogenic specialization of *P. capsici* on the various pepper cultivars grown in Algeria for a long period. Similar supported findings were reported by various investigations in the same line (Pochard and Daubèze, 1980; Yang et al., 1989; Kim and Hwang, 1992). In addition, the cultivar / isolate compatibility may be proved by the disease severity and symptoms development velocity. In resistant cultivars, disease symptoms appear with a slower rate as compared to the susceptible cultivars. Differences in genetic background of pepper cultivars is a direct factor for different resistance response expressed (Pochard et al., 1976).

Conclusion

The late blight disease caused by *P. capsici* Leon is characterized by its severity on sweet pepper (*C. annuum* L) cultivars. The artificial inoculation of various organs of some commercial pepper cultivars by Algerian *P. capsici* isolates in order to select the resistant ones provides that there was a relationship cultivar-isolate. Italico II cultivar showed high resistance to the studied fungal isolates. But under the same conditions, "Esterel" proved to be the most susceptible one. In conclusion, our data suggest that there are different interactions between *P. capsici* isolates and some pepper cultivars at normal plant growth stage. This study demonstrates that information from one geographic area may not accurately predict the response of a resistant cultivar used in another region, including diverse geographic regions within a single state.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Drying of enzyme immobilized on eco-friendly supports

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Endophytic fungus *Cercospora kikuchii* lipase was immobilized on agroindustrial by-products and dried by oven, freeze and spray drying. Spray drying showed the best performance regarding the drying technologies evaluated. Microcrystalline cellulose and rice husk showed the best result since they retained almost 100% of lipase activity after drying. Immobilized derivatives obtained had decreased enzyme activity (≈ 30.0%) during a storage period of six months; and retained an average of 50.0% of the initial activity after five reuse cycles. Water content in immobilized derivatives varied between 4.2 and 6.1% and the water activities ranged from 0.14 to 0.30.

Key words: Enzyme immobilization, drying, *Cercospora kikuchii*, agricultural by-products.

INTRODUCTION

Lipases (triacylglycerol acylhydrolase - EC 3.1.1.3) are enzymes formerly characterized by the ability to reacting with a wide range of substrate with a high enantio and regio selectivity (Singh and Mukhopadhyay, 2012). In fact, this enzyme has a considerable industrial potential and catalyze a number of useful reactions, such as esterification, transesterification, acidolysis, alcoholysis, aminolysis and resolution of racemic mixtures (Reetz, 2002). According to Adlercreutz (2013), the use of lipases in non-conventional media (for esterification and transesterification reactions) has expanded since the mid 1980s, allowing the efficient use of lipases in different industrial processes, in addition to the traditional hydrolysis reactions. Despite widespread research efforts in academics and industry, the application of enzymes can suffer from several drawbacks like instability towards temperature, pH and shear resulting in limited suitability or shelf life (Cowan and Fernandez-Lafuente, 2011). Moreover, soluble enzymes cannot be easily recovered from reaction medium and hence cannot be reused. These operational problems have been improved steadily over the years through the use of process alterations, genetic engineering or immobilization techniques (Polizzi et al., 2007). The last one is attractive for all types of enzymes, in particular lipases due to its use in organic media, bringing some industrial and economical advantages such as recovery and re-use, greater stability and continuous operation (Adlercreutz, 2013). So far, various carriers and methodologies have been used for enzyme immobilization in order to improve the properties of free enzyme (Castro et al., 2001; Freitas et al., 2010; Pereira et al., 2003; Costa-Silva et al., 2014a). Enzymes

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Abbreviations: *p*-NPP, *p*-Nitrophenyl palmitate; **BSA,** bovine serum albumin; **PDA,** potato agar dextrose; **MCC,** microcrystalline cellulose.

immobilization can be carried out on organic and inorganic supports; and the strategies generally used can be classified into three types: non-covalent adsorption, encapsulation, and covalent attachment, each with their proper advantages and disadvantages (Nisha et al., 2012). Adsorption of enzymes onto a support is one of the most basic methods of enzyme immobilization. It involves physical surface interactions between the enzyme and the support matrix and can be driven by combined hydrogen bonding, electrostatic attractive forces, and hydrophobic effects (Adlercreutz, 2013). This immobilization method is important as leaching and excessive denaturing is reduced because it can be conducted under mild conditions. Generally, the choice of a suitable immobilization strategy is determined by the physico-chemical properties of both supporting surface and the enzyme of interest (Khan and Alzohairy, 2010). In this context, the properties of the carrier materials have significant influence on enzyme immobilization. The supports should be readily available, nontoxic, resistant to mechanical stress and should offer a good biological compatibility for enzyme (Zhang et al., 2013). Researchers have used eco-friendly supports like coconut fibers, rice straw, wood cellulignin, chitin, cotton cloth and olive pomace having good results regarding enzyme stability and industrial application features (Brígida et al., 2008; Castro et al., 2001; Synowiecki et al., 1987; Shu et al., 2011). So, eco-friendly supports, mainly of biological origin, not only prevent emergence of ethical issues, but also cut down the production costs (Datta et al., 2013).

The performance of immobilized enzyme relies on several key factors including immobilization strategy, immobilization carrier materials, enzyme pre-treatment and enzyme loading (Zhang et al., 2013). Moreover, dehydration of the enzyme support systems is another important factor that affects the end properties of the immobilized enzyme. The water concentration, even when using organic solvents as reaction media, is a very important parameter that should be measured and adjusted before the immobilized enzyme application. For special case of lipases, water can promote negative effects on the rate of catalysed reactions due to: interference with substrate binding (inhibition), formation of a diffusion barrier for hydrophobic substrates and causes hydrolysis which competes with esterification or transesterification reaction (Adlercreutz, 2013). Several drying technologies can be utilized for the production of dried thermosensitive products like plant extracts, fruit juices, blood products, microorganisms, and enzymes, including freeze drying, spray drying, and spouted and fluidized bed drying (Costa-Silva et al., 2011; Souza and Oliveira, 2005; Samborska and Witrowa-Rajchert, 2005; Bott et al., 2010; Schutyser et al., 2012; Yang et al., 2012). Great attention has been paid to spray drying, a mild and cost-effective convective drying method, albeit in industrial practice for example freeze drying or freezing are often preferred dehydration method for biologics (Schutyser et al., 2012).

The aim of this work was to investigate the potential of several agricultural by-products as low-cost and eco-friendly supports for immobilization of lipase produced by endophytic fungus *Cercospora kikuchii* using the adsorption method followed by dehydration in different drying technologies: oven, freeze drying and spray drying. Thus, exploiting novel immobilization methods and carrier materials have an important significance on enzyme immobilization technology.

MATERIALS AND METHODS

Bradford reagent, *p*-nitrophenyl palmitate (*p*-NPP), bovine serum albumin (BSA), was purchased from Sigma-Aldrich (St. Louis, MO - USA); Potato Agar Dextrose (PDA) was purchased from Biolife (Milan, Italy). Microcrystalline cellulose (MCC) was purchased from Blanver Farmoquimica Ltda (Itapevi, Brazil). All other chemicals, media, and reagents were of analytical grade.

Microorganism and lipase production

The lipase used as model in this study was produced by the endophytic fungus *C. kikuchii,* isolated from *Tithonia diversifolia*. The lipase production was carried out in 250 mL Erlenmeyer flasks containing 100 mL of Vogel's minimum medium supplemented with 2% soybean oil as the only carbon source (Vogel, 1956). The culture was incubated at 30°C in a rotary shaker at 120 rpm for 6 days and the mycelium obtained was removed by vacuum filtration through filter papers (No. 1 Whatmann filter paper, GE Health Care, São Paulo, Brazil) (Costa-Silva et al., 2011). The *C. kikuchii* lipase was biochemically characterized according to Costa-Silva et al. (2014).

Protein assay

Protein concentration was determined according to Bradford method, which involves the binding of Coomassie Brilliant Blue G-250 to protein. Bovine serum albumin was used as a standard (Bradford, 1976).

Enzymatic activity of the free and immobilized lipase

ρ-nitrophenyl palmitate (p-NPP)

Lipase activity assay was performed using ρ-nitrophenyl palmitate (*p*-NPP) as substrate according to Mayordomo et al. (2000). In brief, the reaction mixture consisted of 205 µL of buffer (200 mg of Triton X-100 and 50 mg of gum arabic in 50 mL of 50 mm phosphate buffer, pH 6.5), 45 µL of substrate (15 mg of *p*-NPP in 10 mL of 2-propanol), and 250 µL of enzyme solution (5 mg_{prot}/mL). The mixture was incubated at 40°C for 30 min and then 0.5 mL of 2% trizma base was added. The optical density was measured at 410 nm. Enzymatic activity is given as µmol of *p*NP produced per minute per mg of enzyme (IU) under the conditions described above.

Olive oil emulsion

C. kikuchii lipase activity was also measured using an olive oil

emulsion as substrate, according to the method described by Andrade et al. (2014). The substrate was prepared by mixing 50 g olive oil with 150 g Arabic gum solution (3 wt.%). The reaction mixture containing 5 mL emulsion, 5 mL 0.1 M phosphate buffer (pH 6.5), and immobilized (2.0 g to 2 mg of protein g^{-1} of support) or soluble (0.250 mL - 5 mg_{prot}/mL) lipase was incubated for 5 min at 40°C. The reaction was stopped by addition of 10 mL commercial ethanol. The fatty acids formed were titrated with 0.02 M sodium hydroxide solution in the presence of phenolphthalein as indicator. One international unit of activity was defined as the amount of enzyme that liberates 1 µmol free fatty acid per minute per mg of enzyme (IU) under the conditions described above.

Support

"In natura" Agricultural byproducts supplied by local farmers, were ground and sieved to obtain particle sizes between 50 and 150 mesh. These materials were then washed with distilled water and dried at 60°C before being used as the support matrix. Microcrystalline cellulose (MCC) was also used as a model support.

Support characterization

The specific surface area of supports and total volume and average pore diameter were determined on a Quantachrome equipment New Model 1200, equipped with software for data analysis from measures adsorption-desorption of N_2. Before analysis, samples were subjected to heat treatment at 60°C under vacuum for 48 h to remove the water adsorbed during handling and possible condensate existing in the pores of the solids. The surface areas of the samples were calculated by the Brunauer, Emmett and Teller (BET) method and pore parameters were determined based on calculations BJH (Barrett-Joyner-Halenda). The technique encompasses external area and pore area evaluations to determine the total specific surface area in m^2/g yielding important information in studying the effects of surface porosity and particle size in many applications. The specific surface area of a sample is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface (Fagerlund, 1973). The determination is usually carried out at the temperature of liquid nitrogen. The amount of adsorbed gas is dependent on its relative vapour pressure and is proportional to the total external and internal surface of the material (Fagerlund, 1973). The porosity, pore size distribution and density of the adsorbent material were obtained by mercury porosimetry (Autopore II brand Micromeritics) (Bedin et al., 2013; Ramos et al., 1998).

Lipase immobilization

Lipase was immobilized by adsorption in byproducts in the presence of polyethylene glycol (PEG 1500 MW) as stabilizing agent. Lipase-support (5 mg of protein.g^{-1} of support) system was maintained in contact for 1 h at room temperature under 250 rpm. Hence, the immobilized derivatives were dried by spray drying, oven and freeze drying methods. The activity retention (R_{AE} %) was calculated following the equation 1:

$$\text{RAE}\ (\%) = 100\ \text{x}\ \frac{\text{Immobilized enzyme activity}\ \left(\frac{\text{U}}{\text{mg}}\right)}{\text{Soluble enzyme activity}\ \left(\frac{\text{U}}{\text{mg}}\right)} \quad (1)$$

The immobilization efficiency, IE (η%), was determined by equation 2 (Menoncin et al., 2009), where P0 was the protein content in the lipase solution (mg) and P1 was the amount of protein adsorbed on the supports (mg). P1 was estimated by the difference between total protein content added to immobilization process and the protein content washed from the supports.

$$\text{IE}\ (\%) = 100 \times (\text{P0/P1}) \quad (2)$$

Spray drying

Immobilized derivatives were dehydrated in a bench-top spray dryer (model SD-05, Lab-Plant, Huddersfield, U.K), with concurrent flow regime. The drying chamber has 215 mm in diameter and height of 500 mm. The main components of the system are a feed system of the drying gas, constituted by a blower and an air filter; a temperature control system of the drying gas and a product collect system (cyclone). The enzyme solution (soluble enzyme + supports: 5 mg of protein g^{-1} of support at pH 6.7) was fed to the spray dryer through a feed system, constituted by a peristaltic pump, a two fluid atomizer (inlet orifice diameter of 1.0 mm) and an air compressor. The spray drying conditions were determined in a previous study of optimization of spray drying of crude lipase extract (Costa-Silva et al., 2011). The feed flow rate of atomizing air was set in 17.0 L/min at pressure of 1.5 kgf/cm^2 (Costa-Silva et al., 2014b). The flow rate of the drying air was maintained constant at 60 m^3/h. The drying operation started with injection of the drying air into the SD-05 spray dryer. The air was heated to the desired temperature (100°C) and then the enzyme-support solution was fed at a preset flow rate together with the atomizing air. Measurements of the outlet gas temperature, T_{go}, were taken at regular intervals in order to detect the moment when the dryer attained the steady state (± 15 min).

Freeze-drying

The experiments were performed with a vertical freeze-dryer (SNL 108 B – Thermo Fischer Scientific). Initially, the enzymatic solution (soluble enzyme + supports: 5 mg of protein g^{-1} of support at pH 6.7) was maintained in contact for 1 h at room temperature at 250 rpm. Then the immobilized derivative was frozen in a freezer (refrigerator) at -80°C and posteriorly was submitted to freeze drying. The chamber temperature was maintained at approximately -50°C and 0.05 mbar. The frozen samples were lyophilized for 24 h.

Oven drying

The drying operations were performed using an oven dryer model: Fanem, mod. 315 SE – Guarulhos, Brazil. The enzymatic solution (soluble enzyme + supports: 5 mg of protein g^{-1} of support and pH 6.7) was maintained in contact for 1 h at room temperature at 250 rpm. Then the immobilized derivative was recovered and posteriorly was submitted to oven drying at 40°C for 24 h.

Dryer performance and product properties

Samples of the dried product, using all drying equipment, were collected and used to evaluate the dryer performance and product properties through the following procedures:

Enzymatic activity

The lipase activity assay was performed using *p*-NPP as the substrate, with some modifications (Mayordomo et al., 2000). The

Table 1. Physical characteristics of supports used for lipase immobilization.

Support	As (m^2/g)	At_{pore} (m^2/g)	Dm_{pore} (nm)	ε (%)	ε_{sup} (%)	ρ_{bulk} (g/mL)	ρ_{ap} (g/mL)	ρ_{real} (g/mL)
Sugarcane bagasse	111.1	1.5	155.7	93.7	48.5	0.15	0.8	1.6
Green coconut fiber	166.5	1.5	182.7	83.3	28.9	0.35	1.4	2.1
Rice husk	175.5	1.5	142.1	92.5	50.9	0.24	1.2	3.3
MCC	147.0	1.2	149.2	88.4	45.2	0.45	2.1	3.9
Corn stover	149.1	1.3	129.9	83.4	47.6	0.44	1.4	2.7
Corn cob	163.2	1.4	145.2	86.4	49.1	0.42	1.6	3.1

MCC: microcrystalline celulose; As: specific surface; At_{pore}: total pore area; Dm_{pore}: mean pore diameter; ε: total porosity; ε_{sup}: support porosity; ρ_{bulk}: bulk density; ρ_{ap}: apparent density; ρ_{real}: real density.

difference was the use of 1 g immobilized derivative (5 mg of protein g^{-1} of support) in 50 mM of phosphate buffer, pH 6.5. The solution was used to evaluate the residual activity.

Enzymatic activity of immobilized derivatives after reuse cycles

Residual enzymatic activity was determined for the immobilized lipase derivatives after each batch of reaction (Andrade et al., 2014). The substrate was prepared by mixing 50 g olive oil with 150 g Arabic gum solution (3 wt.%). The reaction mixture containing 5 mL emulsion, 5 mL 0.1 M phosphate buffer (pH 6.5), and immobilized (2.0 g to 2 mg of protein g^{-1} of support) or soluble (0.250 mL to 5 mg prot/mL) lipase was incubated for 5 min at 40°C. The reaction was stopped by addition of 10 mL commercial ethanol. The fatty acids formed were titrated with 0.02 M sodium hydroxide solution in the presence of phenolphthalein as indicator. The residual activity of the biocatalyst was calculated in terms of percentage of activity (U) of the immobilized enzyme measured after each cycle compared with the activity of the immobilized enzyme before the first cycle.

Efficiency of the powder production

The spray-drying performance was evaluated by mass balance, through the determination of the product recovery (R_{EC}), defined as the ratio between the total mass of the product recovered to the mass of enzyme-support composition fed to the system (dry basis).

Product moisture content

The moisture content of the spray-dried product was determined by the oven drying method at 105°C up to a constant weight and was calculated from triplicate analyses and by Karl Fischer method (WHO, 1998; Mendham et al., 2002).

Water activity (Aw)

Water activity was determined in an AQUALAB 4TEV-Decagon according to Norenã et al. (1996).

Enzyme stabilization

The stability of the immobilized enzyme derivatives after spray drying was assessed by monitoring the retention of the enzyme activity during a storage period of 6 months at 5°C.

RESULTS

For industrial use as biocatalysts, soluble enzymes have to be immobilized in order to be reused for several processing cycles. In addition, some other critical enzyme properties need to be improved, including stability, activity, and selectivity. Enzyme immobilization by physical adsorption traditionally refers to binding of the enzymes via weak attractive forces to an inert carrier that has not been chemically modified. Because the carrier is directly involved in binding to the enzyme, both morphologic and chemical characteristics play important roles. Table 1 shows selected physical characteristics of supports used, important for adsorption. The agroin-dustrial by-products showed high specific surface compared to commercial beads (Accurel MP1000: specific surface area was measured at 78.92 cm^2/g or controlled-pore glass beads surface area 22.7 $m^2\ g^{-1}$) which makes them suitable to be used as carriers, particularly for enzymes adsorption studies (Séverac et al., 2011; Gunnlaugsdottir et al., 1998).

The immobilization efficiency was consistent with the values of enzymatic activity retention and the specific surface of the supports evaluated. The coconut husk, corn cobs, rice husk and microcrystalline cellulose showed higher values for the surface area and hence greater immobilization efficiency (Tables 1 and 2) evidencing the existence of a relationship between specific surface and the support adsorption capacity. From the results presented in Table 1 and 2, it can be observed that in general the greater the supports specific surface, the higher is the immobilization efficiency (Figure 1). In this set of experiments, the green coconut fiber, rice husk and corn cob presented the highest values of specific surface and immobilization efficiency.

Although, the knowledge of the sample surface area is important, the pore size distribution is even more critical, since it greatly affects the activity-coupling yield of the biologic immobilization because of the diffusion-controlled phenomena. The samples showed structures with different levels of porosity, with pores diameters larger than 50 nm which classifies them as macroporous materials. When enzyme is immobilized within a porous

Table 2. Comparison between the oven and freeze drying methods applied for immobilization of lipases produced by endophytic fungus *C. Kikuchii* on agroindustrial by-products.

Drying method	Support	IE (%)	R_{EA} (%)	Moisture (%)	A_w (-)
Oven	Sugarcane bagasse	52.0 ± 0.90	74.6 ± 0.61	7.5 ± 0.73	0.24 ± 0.02
	Green coconut fiber	82.0 ± 0.33	71.2 ± 0.76	6.1 ± 0.60	0.35 ± 0.06
	MCC	65.0 ± 0.24	79.6 ± 0.66	5.7 ± 0.85	0.13 ± 0.08
	Rice husk	73.0 ± 0.95	74.2 ± 0.35	5.2 ± 0.32	0.18 ± 0.05
	Corn stover	66.3 ± 0.89	64.9 ± 0.93	7.6 ± 0.40	0.33 ± 0.02
	Corn cob	77.0 ± 0.87	71.2 ± 1.01	7.2 ± 0.25	0.31 ± 0.02
Freeze drying	Sugarcane bagasse	55.0 ± 0.80	75.03 ± 0.21	5.8 ± 0.53	0.29 ± 0.02
	Green coconut fiber	77.5 ± 0.73	75.50 ± 0.50	6.7 ± 0.61	0.35 ± 0.03
	MCC	62.3 ± 0.90	90.73 ± 0.38	4.6 ± 0.32	0.19 ± 0.03
	Rice husk	81.4 ± 0.24	91.13 ± 0.90	4.1 ± 0.12	0.18 ± 0.08
	Corn stover	65.3 ± 0.81	83.03 ± 0.91	7.6 ± 0.20	0.30 ± 0.02
	Corn cob	78.1 ± 0.27	87.97 ± 0.35	6.3 ± 0.25	0.38 ± 0.04

MCC: microcrystalline celulose; IE: immobilization efficiency; R_{EA}: residual lipase activity; A_w: water activity.

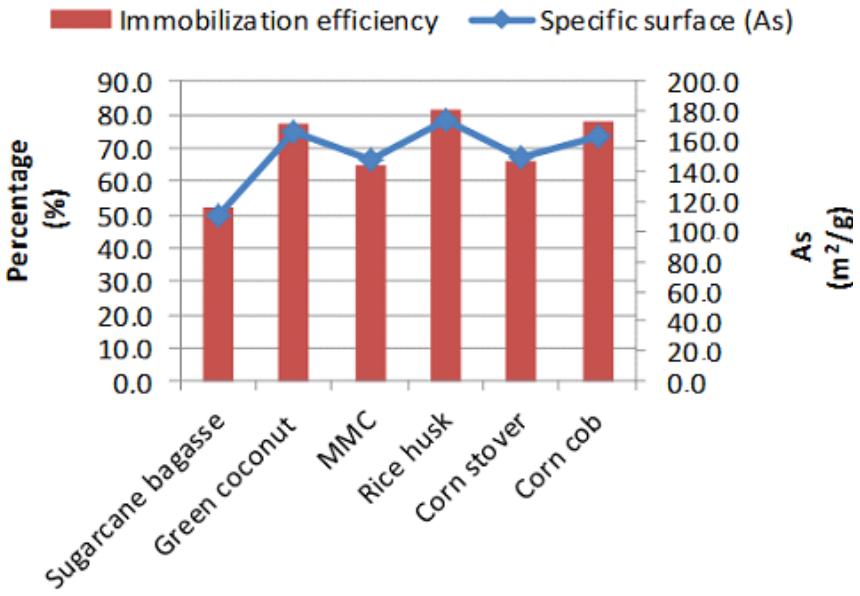

Figure 1. Comparison of immobilization efficiency (η%) and specific surface for samples of lipase immobilized on agricultural byproducts by adsorption.

support, there could also present resistance to internal diffusion, since it must diffuse through the pores in order to contact the biocatalyst, in addition to external mass-transfer effects. Decreasing the dimensions of the porous support containing the biocatalyst can contribute to reduce this additional effect, since the path length the substrate should pass through is significantly reduced, leading to a decrease in the substrate concentration gradient (Soares et al., 1999). For the present work, it was used as a substrate with lower molecular weight, *p*-NPP, and higher enzyme activity retention was obtained compared with olive oil as substrate. In literature the lignocellulosic material density is characterized by an inhomogeneous value. This is understandable since the variations of the characteristics are determined by many factors, such as moisture, particle size, influence the milling mechanism, structure composition, among others. It is important to know the particles density given that it is coupled with the porosity of the sample and, therefore, with the adsorption potential. Besides, it is also important in the productive chain mainly due to fluid dynamic behavior, and product behavior during transport and

Table 3. Effect of spray drying on process yield, residual lipase activity and water content of lipases produced by endophytic fungus *C. Kikuchii* on agroindustrial by-products.

Support	R_{EC} (%)	R_{AE} (%)	Moisture (%)	A_w (-)	Storage R_{AE} (%)	Reuse R_{AE} (%)
Sugarcane bagasse	42.0	94.3 ± 0.67	4.5 ± 0.33	0.14 ± 0.06	72.5 ± 0.33	57.7 ± 0.52
Green coconut fiber	77.0	85.5 ± 0.60	6.1 ± 0.60	0.30 ± 0.01	75.4 ± 0.89	52.1 ± 0.43
MCC	55.0	96.4 ± 0.87	4.2 ± 0.32	0.18 ± 0.03	71.3 ± 0.23	50.9 ± 0.22
Rice husk	60.0	98.6 ± 0.56	5.7 ± 0.55	0.23 ± 0.08	70.4 ± 0.68	51.5 ± 0.33
Corn stover	41.3	93.8 ± 0.40	5.6 ± 0.70	0.30 ± 0.02	74.1 ± 0.96	50.2 ± 0.76
Corn cob	67.0	90.5 ± 0.57	5.2 ± 0.45	0.21± 0.04	76.7 ± 0.13	56.6 ± 0.64

MCC, microcrystalline celulose; R_{EC}, Product recovery (%); R_{EA}, residual lipase activity; A_w, water activity.

storage.

One of the main concerns of enzyme drying is the retention of enzyme activity, which must be retained during all product shelf life. In this work, we combined the advantages of the drying and immobilization processes and made them a unique step using a spray dryer (that showed the best result for enzyme activity retention), making industrial-scale application an economically feasible process. Table 2 shows the effects of the oven and freeze drying methods on properties of the final product. The residual enzymatic activity of the product generated by both drying procedures was in the range of 67.9 to 91.1%. The freeze drying method showed the best results for the residual enzymatic activity. The rice husk was the best support used, maintaining 91.1% of activity after drying. This was followed by microcrystalline cellulose (90.7%), corn cob (87.9%) and corn stover (83.0%). The utilization of moisture as a quality indicator is of particular interest, because water is a key determinant of both the integrity of the solid matrix and support–protein interactions. Water content in the obtained powders ranged between 4.1 and 7.6%. These low values are important since the dehydration could provide an acceptable protein shelf life, and protect the biological activity of these molecules (Namaldi et al., 2006). Water activity is another factor that affects the enzyme stability. Higher values of water activity could provide feasible conditions for microorganism growth and occurrence of degradation reactions. The water activities of the immobilized derivatives were in the range of 0.13 to 0.38; which are considered safe to avoid micro-organism development (Beauchat, 1981).

The spray drying was the third method evaluated. This drying process is a mild technique due to its very short drying times and the relatively low temperatures to which the product is exposed mainly when compared with others convective air-drying methods (Mazza et al., 2003). Table 3 shows the results of product recovery, residual lipase activity, water content, storage stability and enzyme activity after reuse cycles of the spray dried immobilized derivatives. The products recoveries were in the range of 41.3 to 77.0%, which are common values for bench-top spray dryers. Cyclone efficiency and powder deposition in the spray-drying chamber contributes to product loss. The residual enzymatic activity after spray drying was in the range of 85.5 to 98.6%. Therefore, spray dryer was the best drying equipment used for immobilized derivatives dehydration, in terms of retention of enzyme activity. Among all support evaluated, microcrystalline cellulose and rice husk showed the best result because it maintained almost 100% of activity after drying. For the natural lipase substrate, olive oil, the average of enzyme activity retention was 72.7%. Lipase immobilized on microcrystalline cellulose showed the best results, presenting 78.1% of the original activity after spray drying, followed by rice husk (73.9%), green coconut husk (71.3%), sugarcane bagasse (70.7%) and corn cobs (67.8%). The reason for this lower activity could be due to substrate nature: the oil chain is higher than *p*NPP chain, so the access to the biocatalyst is hampered compared with the synthetic substrate.

During spray drying the rapid changes in droplet temperature and moisture content has influence on enzyme conformation and consequently its activity. Other possible stress factors that the protein experiences during spray drying are: adsorption, shearing stress and liquid/air interfacial expansion (Lee, 2002). However, optimum drying conditions and tailored matrix formulations are required to avoid severe structural damage of enzyme chain leading to loss in enzyme activity. In this study, the positive interaction between the lipase and supports during the drying process could be responsible for the high enzyme activity retention. In our previews study, the effect of spray drying conditions on the retention of enzyme activity of lipase, in the presence of carbohydrates have been investigated. The residual enzyme activity after drying with 10% (w/v) of lactose, b-cyclodextrin, maltodextrin, mannitol, gum arabic, and trehalose ranged from 63 to 100% (Costa-Silva et al., 2011). The enzyme activity was lost in the absence of adjuvants. Therefore, the addition of some drying adjuvants (or supports/carries) offers a way to prevent direct contact of enzyme with the high-temperature air and is one of the key techniques for the encapsulation of pharmaceutical enzymes by spray drying.

Stability tests were performed for all spray dried samples,

which were stored at 5°C for up to 6 months. The immobilized derivatives obtained had decreased enzyme activity with an average of only 30.0%, whereas the free enzyme form lost 85.8% of its initial activity in the same period. These results are an indicator of the feasibility of using the spray drying as a way to protect the enzyme properties and to control their stability. The ability to reuse the biocatalyst is of practical and economical importance. In this work, the operational stability of immobilized derivatives was determined using olive oil as the substrate.

The results are also summarized in Table 3. It can be observed that the biocatalysts prepared retained an average of 53.2% of the initial activity after five reuse cycles. Lipase immobilized on sugarcane bagasse showed the best results of operational stability, presenting 68.1% of the original activity after first activity cycle, followed by 63.9% (cycle 2), 61.1% (cycle 3), 58.9% (cycle 4) and 57.7% after five reuse activity cycle. In general, low values of moisture content (and water activity as well) are excellent for product stability. Water content in the obtained powders varied between 4.2 and 6.1% and the water activities of the dried immobilized derivatives were in the range 0.14 to 0.30. The presence of water can accelerate degradation reactions in the solid state, such as deamidation, oxidation, disulfide cross-linking, and Maillard reactions. In particular for proteins, water can affect a complex matrix of protein movements, ranging from oscillatory and rotational motion of individual amino acid groups, to segmental and internal fluctuations that increase their dynamic mobility and thereby decrease their conformational stability (Bone, 1994). Besides, another important observation about drying process is that the water content of immobilized enzymes could be associated with their application. Industrially, lipases are applied mainly in organic reactions and the major of these processes must be performed in the absence of water.

Conclusion

A practical simultaneous immobilization and drying method to load lipase onto non-conventional supports was developed. In this work, it was demonstrated that cheap eco-friendly supports were biocompatible with lipases, rendering immobilized derivatives with characteristics similar to or even better than those previously obtained with natural and synthetic polymers, such as chitosan and silica matrices. It was also demonstrated that spray drying can be successfully used for drying thermally sensitive materials, such as immobilized enzymes, considering the high relative enzymatic activity achieved after the dehydration step. Thus, the procedures established in this paper have promising capability to be applied for immobilization of other enzymes of industrial interest.

Conflict of interests

The authors did not declare any conflict of interest.

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Purification and characterization of phenoloxidase from immunized haemolymph of *Schistocerca gregaria*

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Phenoloxidase (PO) is a key factor in insect immunity. On invasion of microorganisms and pathogens, prophenoloxidase (PPO) changes to its active form, PO. The present study has been conducted to purify and characterize the PO from the haemolymph of desert locust, *Schistocerca gregaria* (Forskal) following activation of immune system by invasion of bacteria, *Bacillus thuringiensis kurstaki* (*Bt*). PO is purified by a combination of ammonium sulfate precipitation, blue sepharose CL-6B and phenyl sepharose CL-4B chromatography yielded a 209.97-fold purity and 54.75% recovery of activity. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) reveals that the molecular weight of the purified PO is 70.154 kDa. The purified PO is characterized in terms of its biochemical and enzymatic properties by using L-DOPA as a specific substrate. Ca^{2+} and Cu^{2+} significantly stimulated PO activity when compared with other metals. The PO reaction was strongly inhibited by phenylthiourea and thiourea, moderately inhibited by ethylene diamine tetractic acid (EDTA) and poorly inhibited by ethylene glycol tetraacetic acid (EGTA) and diethyl dithiocarbamate (DTC). Inhibition of PO showed excellent recovery ability by addition of Ca^{2+} on EGTA-inhibited enzyme. Therefore, PO is most probably a kind of tyrosinase-type Ca^{2+}-containing metalloenzyme. The content of Ca^{2+} is higher than other trace metal elements. The reactive intermediates yielded by PO with its specific substrate L-DOPA had a broad-spectrum bactericidal activity against Gram +ve bacteria (*Bacillus cereus* and *Staphylococcus aureus)* with a greater degree more than Gram-ve bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). From the present study, PO from *S. gregaria* is most probably a tyrosinase-type calcium-containing mono-phenoloxidase, which functions not only as a catalytic enzyme in melanin production in locusts, but perhaps also as a humoral factor in host defense via melaninization as in other insects.

Key words: *Schistocerca gregaria*, phenoloxidase, purification.

INTRODUCTION

The desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) represents a relatively important group of plant-feeding insects. They have strong immune responses against bacteria, as previously shown by

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(Meshrif and Barakat, 2002; Barakat et al., 2002; Mo'men et al., 2010). The high interest in biological control means of controlling insect pests intensifies the need for investigating the response of insect to disease organisms. The haemolymph offer a readily accessible criterion of this response. The last and more important line of defense is the internal defense system that is comprised of cellular components and humoral mechanisms that offer a very effective protection against invading microorganisms and co-operatively interact to destroy non-self-elements. The cellular defense by haemocytes takes place immediately after contact with the foreign invader (Carton and Nappi, 1997). Humoral mechanisms that are essentially concerned with the ability of insect to recognize and dispose self from non-self, and involve several physiologically active substances, normally present in the native haemolymph or synthesized after natural infections (Boman and Hultmark, 1987). These substances appear in the haemolymph within a few hours after infection and display a broad spectrum of antimicrobial activity. Phenoloxidase (PO) is one of the most important enzymes that involved in the innate immune system of invertebrates. PO is synthesized as an inactive zymogen, prophenoloxidase (PPO) which can be activated by specific proteolysis (Cerenius and Söderhäll, 2004). When insects are infected by microorganisms, PPO activation elicits by microbial cell surface components, such as, lipopolysaccharide (LPS), peptidoglycans, β-1,3-glucose (Mo'men et al., 2012), the activities of the haemocytic enzymes, including phenoloxidase are enhanced during the challenge course.

However, due to the instability and rapid loss of the activity of this enzyme during the purification, more attention is paid to the investigation of PPO. So far, PPO is purified and characterized from only a small number of insect species including Lepidopteran, *Hyalophora cecropia* (Andersson et al., 1989) and *Ostrinia furnacalis* (Feng et al., 2008). Dipteran, *Sarcophaga bullata* (Chase et al., 2000), and cockroaches (Durrant et al., 1993).PO activity is investigated in other insects; *Eurygaster integriceps* (Zibaee et al., 2011) and *Hyphantria cunea* (Ajamhassani et al., 2012). Our knowledge of this enzyme (PO) at the protein level is limited. For example, the exact site of synthesis, regulation of PPO, its activating enzymes and inhibitors are still controversial. Although, there have been a number of studies involving various functional aspects of insect PPO, one or more of these aspects as integral parts of cells could be liberated to act on bacteria upon destruction of the cells or a change in the cell's natural environment. Accordingly, the amount of antibacterial activity in the blood of normal insect should be proportional to the amount of cell destruction or to the degree in which the environment was altered.

The present study aims to isolate, purify and characterize the components involved in the PO cascade system, and to clarify more information dealing with the physicochemical properties of phenoloxidase of *S. gregaria*.

MATERIALS AND METHODS

Maintenance of insects

The desert locust, *S. gregaria* (Forskal), was maintained and reared for ten generations at 30 ± 2˚C, a photoperiod of 16:8 (light: dark) and relative humidity varied between 60 and 80%, according to methods of Huxham and Lackie (1989). Cages were illuminated with one electric bulb, 100 watt, per cage in winter, and 60 watt in summer. All experiments outlined below were carried out with adults (both sexes), all being within 2 to 4 days after ecdysis.

Source of the bacterial pathogens

The bacterium, *Bacillus thuringiensis kurstaki (Bt*) (3200 IU/mg, AGERIN- wettable powder) was chosen as the pathogen for this study because of its wide use as a biocontrol agent among insects. The bacterium, *B. thuringiensis kurstaki* (*Bt)* were produced by the Agricultural Genetic Engineering Research Institute (AGERI) at the Ministry of Agriculture, Giza, Egypt. Non-Pathogenic strains of Gram positive bacteria (*Bacillus cereus* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), were obtained from Department of Microbiology at Ain Shams University.

Mass culturing of the bacterial pathogens

Subcultures from bacterial pathogens were grown aerobically at 28 ± 2°C in nutrient broth tubes for 48 h. To obtain solitary pure colonies; nutrient agar plates were prepared and cultured with inoculates of the grown bacteria in the nutrient broth, using the streaking dilution method. The plates were incubated at 28 ± 2°C for 48 h. After growth, only solitary colonies were selected, cultured on nutrient agar slants and incubated at 28 ± 2°C for 48 h, and then kept in the refrigerator at 4°C until used. These slants were regenerated monthly.

Injection technique and haemolymph collection

In order to induce activation of locust immune system, which leads to the conversion of prophenoloxidase cascade to its active form (phenoloxidase), a stock suspension of a sub-lethal concentration of *Bt* was prepared, 10 µl of this concentration was injected into the haemocoel of the locusts. Insects were injected with a 10 µl Hamilton micro-syringe fitted with a 26-gauge needle according to Miranpuri and Khachatourians (1993). Ten microlitres of the concentration 1.2×10^6 cells/ml of (*Btk)* were used as a sub-lethal concentration to investigate the subsequent experiments according to Mo'men et al. (2010). Injected locusts were removed from the rearing cages, submerged in hot water bath at 60°C for 2 to 5 min; they were allowed to dry on paper towel. The heat-killed insects were amputated at the hind coxa with fine scissors. The haemolymph was obtained with a fine-tipped calibrated glass capillary, which was kept at -20°C until further analyses.

Estimation of the total haemolymph proteins

The total protein concentration in the haemolymph was quantified according to the method described by Bradford (1976).

Phenoloxidase activity assay

In order to measure PO activity, a preliminary assay was set up, in which we recorded the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) spectrophotometrically at 470 nm, according to Aso et al. (1985) with some modifications. A solution of L-DOPA (2 mg/ml) was made in a sodium phosphate buffer (SPB) (0.01 M, pH 5.9). Aliquots (20 µl) of haemolymph were diluted (v/v) in a sodium cacodylate buffer (SCB) (0.01 M sodium cacodylate, 0.25 M sucrose, 0.01 M trisodium citrate), were added to 2 ml of DOPA solution, after which the formation of dopachrome (reddish brown pigments) was recorded each minute for 5 min to make sure that we respected the linear increase of the optical density. In case of control, 20 µl SCB were used. The phenoloxidase activity was expressed as PO unit, where one unit is the amount of enzyme activity required to produce an increase in the absorbance by 0.001 min/mg protein.

Phenoloxidase purification

All purification steps were performed at 4°C in a sodium cacodylate buffer (SCB) unless otherwise noted. Haemolymph (8 ml) was first diluted into 2:1 with the CB. The saturated ammonium sulphate solution was added to haemolymph until reaching a saturation of 40%. The precipitate was spun down by centrifugation for 10 min at 12,000 rpm, and redissolved in 500 µl of 20 mmol/L sodium cacodylate solution (pH 6.5). The protein was dialyzed in 2000 ml CB overnight at 4°C, and then applied to a blue sepharose CL-6B column (1.0 cm × 10 cm) pre-equilibrated with the CB. The column was eluted with an elution buffer (100 mM/L $CaCl_2$, 10 mM/L Na_2CB and pH 6.5) at a flow rate of 1.5 ml/min. The fractions containing PO from three simultaneous blue sepharose CL-6B chromatography were pooled, and concentrated with sucrose, then applied to a phenyl sepharose CL-4B column (0.8 cm × 12 cm) that was equilibrated with the CB buffer. The column was washed with distilled water at a flow rate of 1.5 ml/min until the absorbance of fractions at 280 nm returned to zero. The fractions with PO activity were dried in a Heto FD3 Model Vacuum Cold Dryer. The purified enzyme was stored at − 80°C according to Feng et al. (2008).

Molecular weight estimation

To determine the success of purification scheme we monitor the procedure of each step by performing, one-dimensional gel electrophoresis in vertical polyacrylamide gel; Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970). On 10% gels, using SDS-molecular standard mixture of proteins; 205 kDa: 29 kDa (from Sigma) with a 4% stacking gel, at 100 volts for 5 h at room temperature.

Isolation of PO from polyacrylamide gel

After electrophoresis, the protein band of interest must be located in the gel by Side-strip technique. Staining of strips of the gel cut from the side when isolating abundant proteins that are well separated from other bands, was achieved according to Harlow and David (1988).

Polyclonal antibody production

Specific polyclonal antisera against the isolated PO were raised in young rabbit; approximately 800 µg of the purified PO was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites of the rabbit, at intervals of 4 weeks. Eight days after the final booster, blood was collected and serum prepared according to Fergusson (1996). The antisera were aliquot and stored at -70°C.

Conformation of antibody specificity

It was carried out by using Western blotting analysis. This technique, as described by Towbin et al. (1979), depends on placing a sheet of nitrocellulose against the surface of an SDS-PAGE protein fractionation gel, and then a current is applied across the gel, thus causing the protein to move out of the gel onto the nitrocellulose where they bind firmly.

Effect of metal ions on the PO activity

To verify if the activation of the purified PO is influenced by the presence and concentration of metal ions, the activity of PO in the presence of different metal ions; $MgSO_4$, $ZnSO_4$, $MnCl_2$, $CuSO_4$ and $CaCl_2$, was measured, respectively. According to methods of Feng et al. (2008), the purified PO (10 µg) was added to solutions diluted, respectively, with 100 mmol/L $MgSO_4$, 100 mmol/L $ZnSO_4$, 100 mmol/L $MnCl_2$, 100 mmol/L $CuSO_4$, and 100 mmol/L $CaCl_2$ to various concentrations. 40 µl of 0.1 mol/L sodium phosphate buffer (SPB) and 100 µl of 2 mmol/L L-DOPA were added. The mixtures in a final volume of 1 ml were incubated for 30 min at 30°C, and the increase in absorbance at 490 nm after 10 min was continuously monitored for calculation of the PO activity.

Inhibition assay of PO activity

In order to determine the effect of various inhibitors on the activity of the purified PO, different compounds including: thiourea, phenylthiourea, ethylene diamine tetraacetic acid (EDTA), diethyldithiocarbamate (DTC) and ethylene glycol tetraacetic acid (EGTA), were tested for their inhibitory effect according to the method described by Fan et al. (2009).

Recovery effect of PO activity

To verify the metalloenzyme property of the PO, the recovery effects of some metal ions on PO activity were investigated according to the method of Fan et al. (2009). Recovery effect of Ca^{2+} on the activity of ethylene glycol tetraacetic acid (EGTA) pretreated purified PO from *S. gregaria* haemolymph, measurement and comparison between the enzymatic activity of 10 µl purified PO only, purified PO + 20 mM EGTA, purified PO + 20 mM EGTA + 10 mM Ca^{2+}, purified PO + 20 mM EGTA + 15 mM Ca^{2+} and purified PO + 20 mM EGTA + 20 mM Ca^{2+} were made; and the reaction mixture was measured spectrophotometrically under the same conditions as described above.

Substrate specificity assay of PO

We investigated the substrate specificity of PO purified from the haemolymph of locusts using the method according to Andersen (1980).

Effect of reactive intermediates produced in PO-catalyzed reactions

In order to test the effect of PO-substrate-derived compounds on the growth and survival of bacterial cells: *Bacillus cereus, S.*

Table 1. Total haemolymph protein concentration and PO activity of unpurified and purified haemolymph of *S. gregaria,* following activation of immune system with *Bt* injection.

Haemolymph sample	Total protein Concentration (mg/ml) [A]	PO activity (U/mg)[B]		Recovery (%)[E]	Purification fold [F]
		Specific activity [C]	Total activity [D]		
Unpurified	80.537± 0.07**	50.163 ± 1.01**	4039.97±3.6*	100	1
Purified step 1	18.304± 2.26*	185.089±0.04*	3387.86±2.1**	83.85	3.68
Purified step 2	5.001± 0.018*	600.03±0.21**	3000.70±0.23**	74.27	11.96
Purified step 3	0.21± 1.45*	10533.01±0.01*	2211.93±2.10*	54.75	209.97
Control	15.03±0.06	0.003± 0.55		---	---

n=3 replicates per test, (Mean ± SE). *Significance (P < 0.05). A, Total protein concentration (mg/ml) determined by dye binding method using BSA as standard protein; B, PO activity measured by L-DOPA (2mg/ml) as a substrate at 470 nm, and expressed in unit/mg protein; C, specific activity; (total activity / total protein of each purification step); D,total activity; (The enzyme activity in the volume of fraction used in the assay x the fraction total volume); E, recover percent (yield); (Total activity retained after each purification step/ total activity of unpurified sample) x 100; Recover percent in the unpurified sample is taken to be 100%. F: Purification fold; (Specific activity calculated after each purification step / specific activity of unpurified sample). Step 1: $(NH_4)_2SO_4$, 40% saturation. Step 2: Blue Sepharose CL-6B chromatography. Step 3: Phenyl Sepharose CL-4B chromatography. **Significant (P<0.01) in compared with appropriate control.

aureus, E. coli and *P. aeruginosa,* (dependent on their growth rates), the experiment was carried out according to Zhao et al. (2007).

Statistical analysis

Results of susceptibility test were represented graphically as probit-logarithmic regression line. Statistical analysis of data was made by using software: Probit Analysis Program, Version 4.0. All data of the rest experiments were expressed as mean ± standard error (SE) and analyzed by using the SPSS11.5.0 software (SPSS Inc., 2012). The differences between means were analyzed by independent samples *t*-test and one-way ANOVA. The level of significance for each experiment was set at P < 0.05 or P < 0.01.

RESULTS

Total haemolymph protein concentration

The unpurified haemolymph of *S. gregaria* adult contained 80.537± 0.07 mg/ml protein. The total protein content of purified haemolymph after serial purification steps using ammonium sulphate precipitation $(NH_4)_2SO_4$, 40% saturation followed by affinity chromatography (Blue Sepharose CL-6B chroma-tography and Phenyl Sepharose CL-4B chromatography) were decreased significantly compared with unpurified haemolymph. Data presented and graphically illustrated in Table (1).

Enzyme purification

The PO purification results are shown in Table 1. The purification procedure yielded a total of 0.21 mg PO from a starting sample of 8 ml haemolymph containing about 80.537 mg total protein. The PO was purified 209.97-fold with a 54.75% total recovery of activity. SDS-PAGE of the purified protein revealed a single band with an estimated molecular mass of approximately 70.154 kDa (Figure 1).

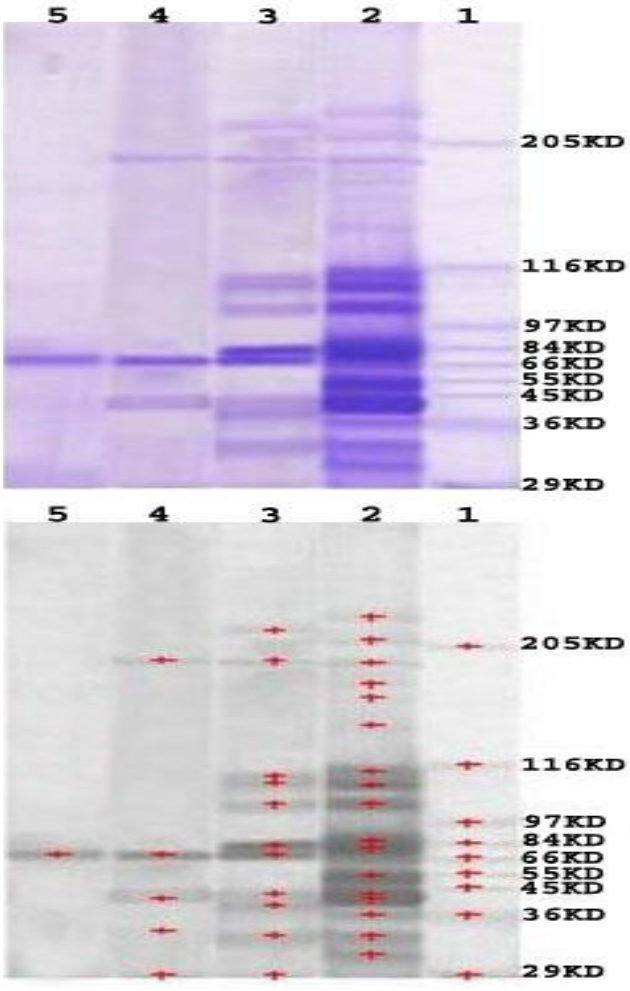

Figure 1. Protein bands of unpurified and purified haemolymph of *S. gregaria* adults determined from SDS-PAGE (10%) method. lane1: Marker of protein; lane 2: haemolymph of *S. gregaria*; lane 3: the precipitation of ammonium sulphate; lane 4: the fractions of Blue Sepharose CL-6B; lane 5: the fractions of Phenyl Sepharose CL-4B.

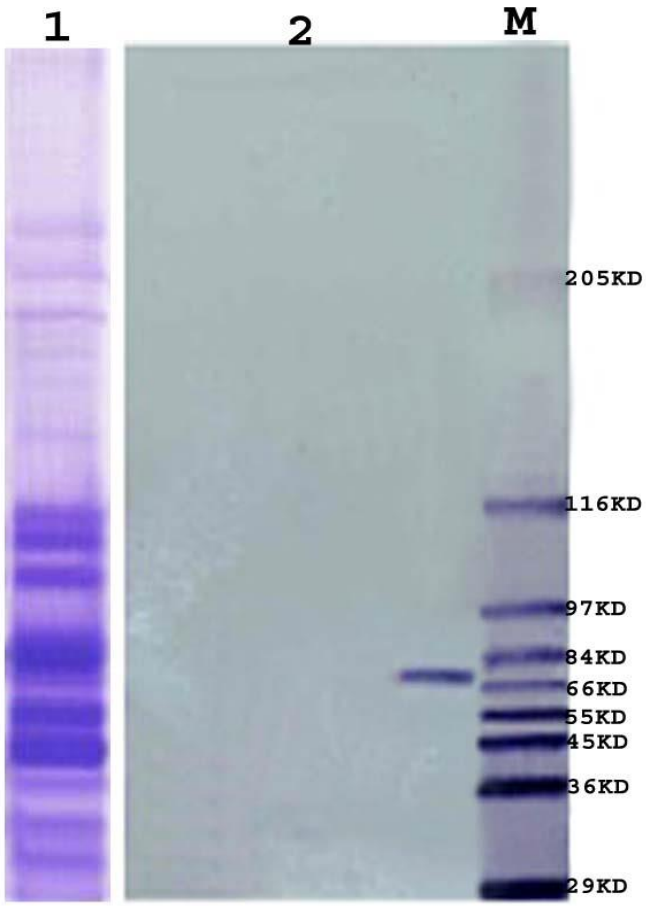

Figure 2. Western blotting analysis of PO purified from the haemolymph of *S. gregaria* adults: Marker of protein; lane 1: haemolymph of *S. gregaria* using SDS-PAGE technique; lane 2: Rabbit antisera against the purified PO.

Confirmation of antibody specificity

Rabbit antisera against the purified PO were obtained. Specific polyclonal antisera against the isolated PO were capable of recognizing one band of approximately 70.154 kDa on SDS-PAGE gels corresponding to that of purified PO band detected from SDS-PAGE (Figure 2).

Effect of metal ions on PO activity

The PO activity was increased when the Ca^{2+}, Mg^{2+} and Cu^{2+} concentration was increased to 15 mM/L. However, when the concentration was increased to 20 mM/L, it became inhibitory. In the presence of Mn^{2+} or Zn^{2+}, the PO activity was increased only by a slight level (Figure 3).

Inhibition assay

The PO activity was completely inhibited by phenylthiourea and thiourea at (20 µM/L), moderately inhibited by ethylendiamine-tetracetic acid (EDTA) and triethylen\ tetraminehexaacetic acid (TTHA), poorly inhibited by diethyldithio-carbamate (DTC) (Figure 4).

Recovery effect of Ca^{2+} on the activity of EGTA-pretreated PO purified from activated *S. gregaria* haemolymph

The enzymatic activity of the PO greatly inhibited by 20mM EGTA was restored to its original level by 15 mM Ca^{2+} .This results indicate that PO purified from *S. gregaria* probably is a calcium-containing metalloenzyme (Figure 5).

Enzyme activity and substrate specificity

In addition to L-DOPA, other O-phenols were suitable as substrates for the *S. gregaria* phenoloxidase, while no activity was detected towards the mono-phenol tyrosine (Figure 6).

Effect of PO and L-DOPA derived compounds on the growth of bacterial cells

After PO and L-DOPA had been incubated with bacterial cells, we observed that the bacterial growth was signifycantly reduced ($P < 0.05$). Bacterial growth was not affected ($P > 0.05$) after the cells had been treated with the substrate or PO alone (Figure 7). The cell mortality was determined to be higher in the bacterial samples (*B. cereus*, *S. aureus, E. coli* and *P. aeruginosa*, respectively) treated with PO and L-DOPA. *B. cereus* was most susceptible, while *P. aeruginosa* was most resistant.

DISCUSSION

Several investigators purified and characterized some different insect POs (Durrant et al., 1993; Chase et al., 2000; Fan et al., 2009; Zibaee et al., 2011; Ajamhassani et al., 2012). Three types of POs are reported in insects as follows: laccase type (E.C.1.10.3.2; p-diphenol: O2 oxidoreductase), catechol oxidase type (E.C.1.10.3.1; diphenol: O2 oxidoreductase), and tyrosinase type (E.C.1.14.18.1; monophenol, L-DOPA: O2 oxido-reductase) (Barrett, 1987). The major problem to analyze the PO is the lack of effective tools to identify and quantitate individual PO isoforms. Several investigators use conventional chromatographic method (Chase et al., 2000) instead of immunoaffinity chromatography which is ineffective for purifying PO (Kopácek et al., 1995). In the present study, a combination of ammonium sulfate precipitation, blue sepharose CL-6B chromatography and

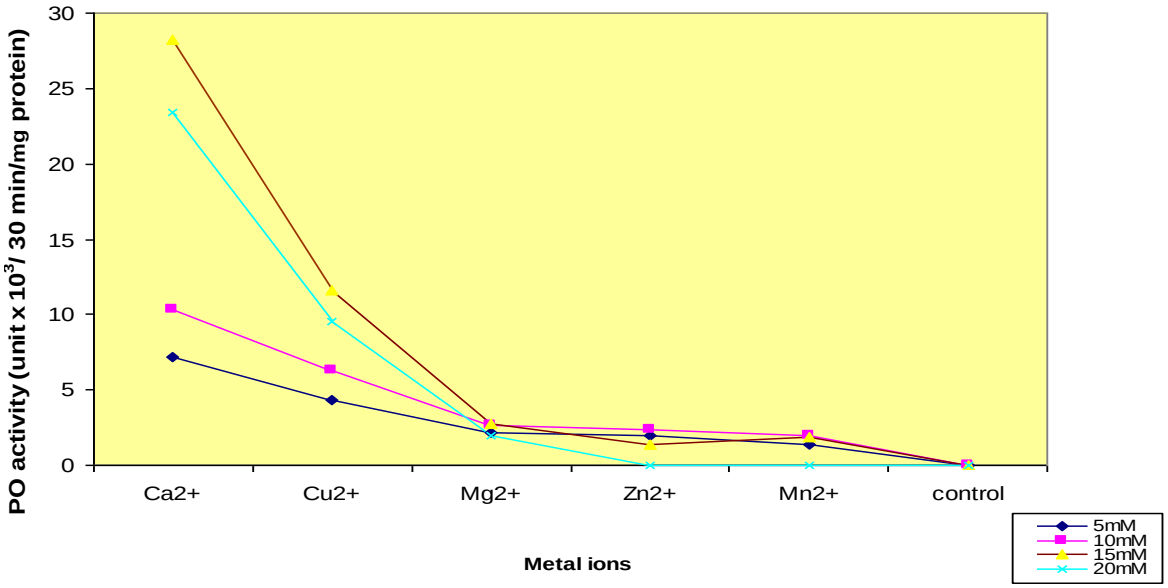

Figure 3. Effect of various metal ions on the activity of PO purified from the activated haemolymph of *S. gregaria* adults.

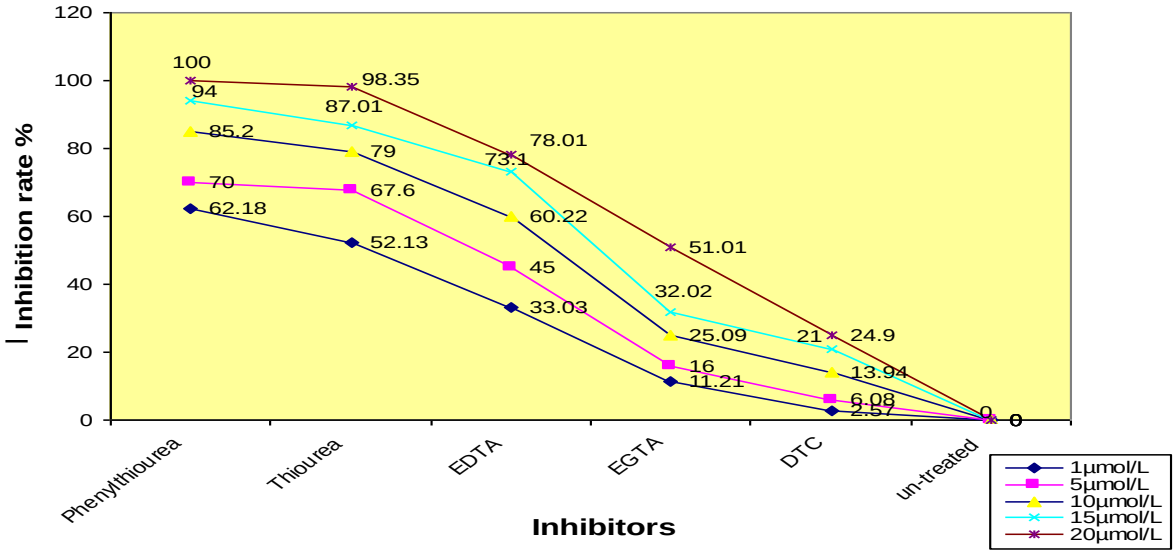

Figure 4. Effect of various inhibitors on the activity of PO purified from the activated haemolymph of *S. gregaria* adults.

phenyl sepharose CL-4B chromatography was employed to purify the PO from the haemolymph of *S. gregaria*. The enzyme estimates as 70.154 KDa by gel filtration in Sepharose and SDS-PAGE. These data are compatible with the purified enzyme from *S. bullata* (Chase et al., 2000), *H. cecropia* (Anderson et al., 1989), *Locusta migratoria* (Cherqui et al., 1996), *E. integriceps* (Zibaee et al., 2011), *H. cunea* (Ajamhassani et al., 2012) and *Helicoverpa armigera* (Goudru et al., 2013), that a single isoform characterizes from them. Many reports detect different isoforms of PO in several insects, for example, there are two isoforms in *Galleria mellonella* (Kopácek et al., 1995) and *Bombyx mori* (Yasuhara et al., 1995), three isoforms in the fruit fly *Drosophila melanogaster* (Fujimoto et al., 1993), and *Branchiostoma tsingtauense* (Pang et al., 2005), six in the mosquito *Anopheles gambiae* (Müller et al., 1999), The physiological significance of PO isoforms in the above mentioned insects

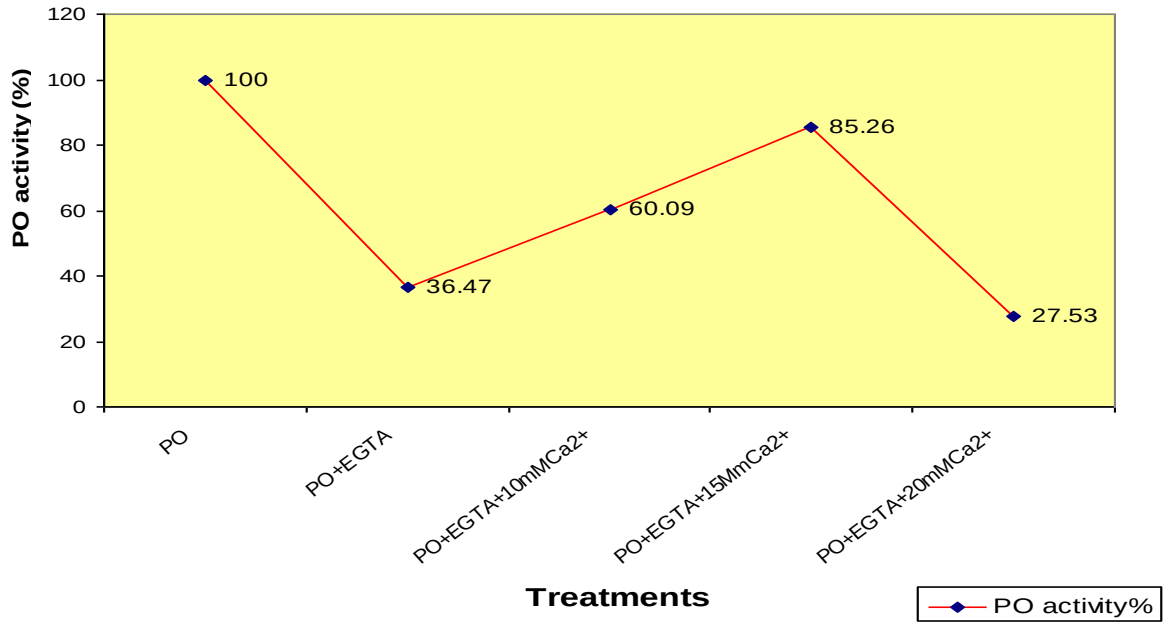

Figure 5. Recovery Effect of Ca^{2+}on the activity of EGTA pre-treated PO purified from the activated haemolymph of *S. gregaria* adults.

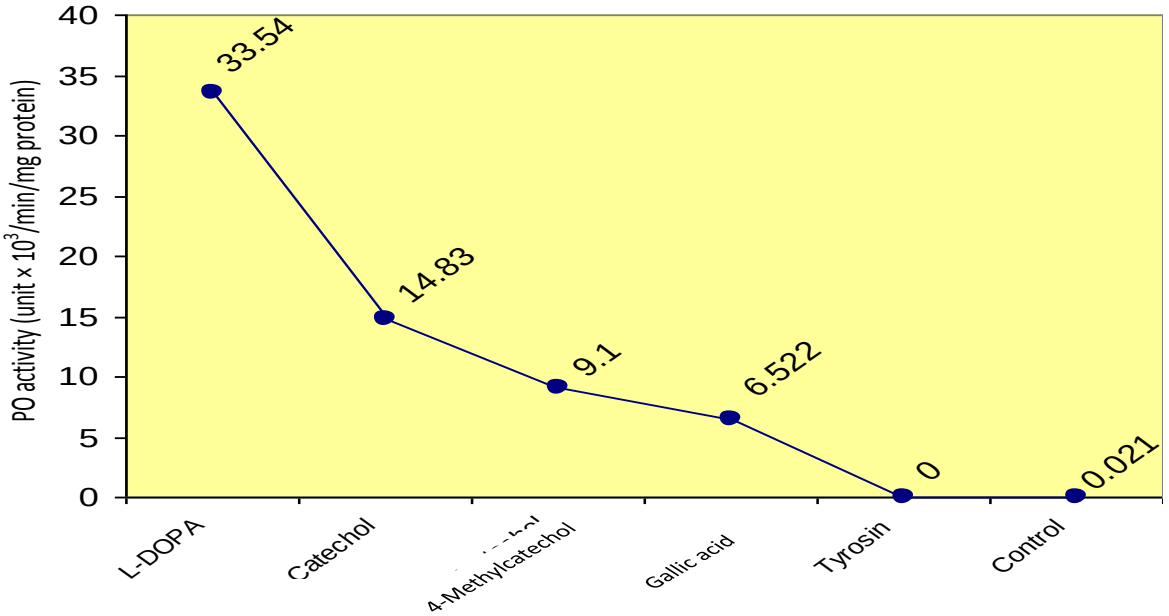

Figure 6. Substrate specificity and the corresponding activity of PO purified from the activated haemolymph of adult *S. gregaria.*

still remains to be studied (Feng et al., 2008). Different substrates can adopt the appropriate conformation to interact with the PO protein. There is no information at this time on the substrate binding pocket in PO. The differences in the substrate binding pockets between the different insects are probably the result of differences in substrate-protein contact points or differences in the size of the substrate binding pocket reference. In the present study, the PO from *S. gregaria* is capable of oxidizing L-DOPA effectively, but fails to oxidize tyrosine. These results implies that this enzyme is most probably a kind of monophenol, tyrosinase-type *o*-oxidoreductase, not a

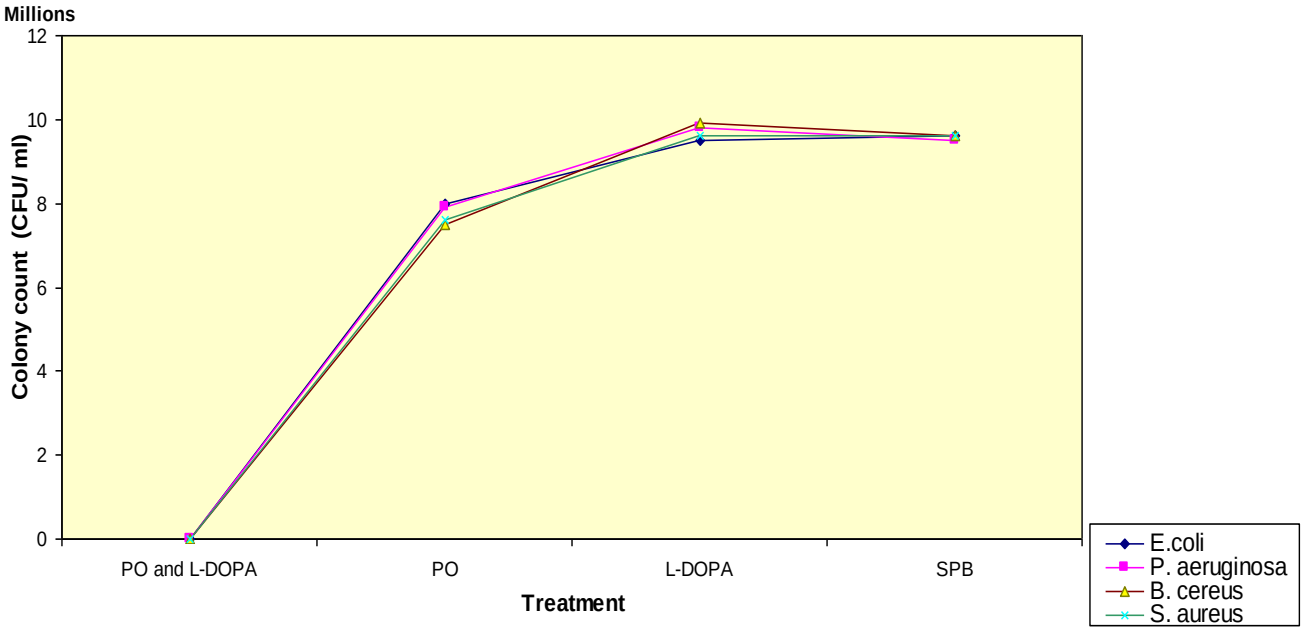

Figure 7. Effect of reactive intermediates (generated *in vitro* by PO purified from the haemolymph of *S. gregaria*) on the growth of Gram +ve and Gram -ve bacterial cells.

laccase-type or catechol oxidase-type enzyme, this is in agreement with the results of Cherqui et al. (1996), Pang et al. (2005) and Asano and Ashida (2001).

Phenoloxidase activity is almost entirely inhibited by phenylthiourea and thiourea. This complete inhibition may be attributed to the influence of phenylthiourea on this process which is caused by its interaction with active sites of PO rather than with intermediate products of DOPA oxidation preventing the subsequent melanin formation. This explanation is in agreement with the results of Ryazanova et al. (2012). Results also indicate that treatment with EGTA nearly showed a similar pattern of inhibition as EDTA, since EDTA is a divalent cation scavenger and EGTA is a specific calcium chelator. Inhibition of PO activity with EDTA, indicates the involvement of divalent cations in the melanin-synthesis pathway, it may resemble other invertebrate POs that contain multiple copper atoms and/or copper binding sites (Aspan and Söderhäll, 1991; Aspan et al., 1995; Nellaiappan and Sugumaran, 1996). In fact, the effects of both EDTA and EGTA may be due to calcium dependency of the POs, as calcium is known to increase activity of several invertebrate POs (Perdomo-Morales et al., 2007), suggesting that the binding of some calcium atoms is necessary in the activating center of *S. gregaria* PO. The result of recovery test show that the enzymatic activity of the purified PO greatly inhibits by 20 mM EGTA, restores to its original level by 15 mM Ca^{2+}. It was concluded that *S. gregaria* PO is most probably a kind of calcium-containing metalloenzyme and different from other insects such as *Heliothis virescens* (Lockey and Ourth (1992). DTC is a specific chelator for cupper presence in PPO which may explain the poor inhibition of *S. gregaria* PO activity that indicates few cupper atoms exist in the *S. gregaria* PO. This explanation is in accordance with those of Feng et al. (2008) on *O. furnacalis* larvae. Some metal ions can significantly modify the structure of PO (Li et al., 2000) that leads to increase or decrease in the activity of the enzyme, this ability to change conformation in solution might explain how the enzyme enhances its activity. Several metal ions tested with PO of *S. gregaria* showed that PO activity increases significantly when the Ca^{2+} concentration increases to 15 mM/L. However, when the concentration increases to 20 mM/L, it became inhibitory. In the presence of Cu^{2+} the PO activity increases only by a moderate level, while Mg^{2+}, Zn^{2+} and Mn^{2+} show non-significant increase in the PO activity. So, it was concluded that the content of calcium is higher than other trace metal elements. Calcium-mediated PO activity enhancement has been reported for a large number of insects: for example *B. mori* (Ashida et al., 1983), *S. gregaria* (Dularay and Lackie, 1985), *Blaberus craniifer* (Leonard et al., 1985), *L. migratoria* (Brehelin et al., 1989), *Lymantria dispar* and *Galleria mellonella* (Dunphy, 1991). Thus, the knowledge of the binding of trace metal elements to the PO is required to be investigated extensively.

Insect POs, or tyrosinase-type POs, are similar to mammalian tyrosinases with two catalytic activities: the oxygenase activity which hydroxylates monophenols to o-diphenols and the oxidase activity which converts o- diphenols

to quinones (Sugumaran, 2002; Nappi and Christensen, 2005). It has long been known that insects rely heavily on tyrosine metabolism for cuticle hardening and for innate immune responses (Vavricka et al., 2014). It was found that the roles of melanization include anti-bacterial, anti-fungal, anti-viral and anti-parasitic responses. This reaction has a broad-spectrum for all possible agents that can invade insects. This kind of universal killing power seems to stem from its basic mechanism of toxicity (Zhao et al., 2007). Several comprehensive reviews covering the humoral immunity discussed a number of immune proteins that were induced by the injection with bacteria (Gtz and Boman, 1985; Boman and Hultmark, 1987; and Hultmark, 1993). Barakat (1997) indicated that the humoral defense reactions needed to some extent for newly synthesis and release of the antibacterial proteins. Meshrif and Barakat (2002) investigated the appearance of antibacterial substances in the haemolymph of the bacterial-injected insects as well as the uninjected insects, the antibacterial activity needs certain time to appear and integrate with the cellular reactions to produce an effective immune response in this species. Therefore, we decided to test the controversial function of PO directly by measuring possible antimicrobial activity of the reactive compounds produced *in-vitro* by this enzyme. After treating bacteria with the reaction mixtures containing purified *S. gregaria* PO with its specific substrate L-DOPA, the antibacterial effect (growth inhibition of bacterial cells) was observed. These findings established that the reactive intermediates yielded by PO had a broad-spectrum bactericidal activity against bacteria. Gram +ve bacteria (*B. cereus* and *S. aureus*) are more susceptible than Gram-ve bacteria (*E. coli* and *P. aeruginosa*). These results are similar to those of Zhao et al. (2007) who reported the antimicrobial effect of reactive intermediates produced in phenoloxidase-catalyzed reactions after being treated with *Manduca sexta* PO and dopamine, *Bacillus subtilis* ceased to grow.

Cerenius et al. (2010) revealed that an active PO isolated from the freshwater crayfish *Pacifistic leniusculus* exhibited a strong antibacterial effect *in-vitro* on the bacteria Gram -ve whereas, a weaker but still significant effect against Gram +ve. Rowley et al. (2011) investigated the possible role of the PPO system of *L. migratoria* in the killing/inhibition of growth of several species of bacteria, and suggesting that the antimicrobial factor(s) may have been generated by either the PPO cascade or a related enzyme system. The limited data gathered so far seem to indicate that certain bacterial species are more sensitive than other to quinone inter-mediates produced in the melanization cascade. These intermediates may have developed a tolerance to the presence of some bacteria. These results established that PPO activation is an integral component of the insect defense system involving a multitude of enzymes (e.g. proteinases, oxidases, and dopachrome conversion enzyme (DCE), which immobilize and kill invading microorganisms. The nature of these bioactive molecules requires detailed study to characterize the significance of these compounds. From the present study, PO from *S. gregaria* is most probably a tyrosinase-type calcium-containing mono-phenoloxidase, which functions not only as a catalytic enzyme in melanin production in locusts, but perhaps also as a humoral factor in host defense via melaninization as in other insects. To understand the similarities as well as differences in molecular characterization and physiological function among these arthropod POs, it is necessary to conduct more accurate, qualitative and quantitative analyses by cloning and transcriptional or translational detection of PO.

Conflict of interests

The author(s) did not declare any conflict of interest.

Abbreviations: EDTA, Ethylene diamine tetractic acid; **EGTA,** ethylene glycol tetraacetic acid; **DTC,** diethyl dithiocarbamate; **AGERI,** agricultural genetic engineering research institute; **L-DOPA,** L -dihydroxyphenylalanine; **SCB,** sodium cacodylate buffer.

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