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Studies on the characterization and distribution of fatty acids and minor components of high-erucic acid mustard oil and low-erucic acid rapeseed oil

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Abstract

The aim of this work was to compare the positional fatty acid composition, sterols, tocopherols and oxidative stability of mustard oil (MO) and rapeseed oil (RSO). The MO contained higher levels of erucic acid (42.8%) and linolenic acid (18.2%), compared with RSO (0.4 and 8.6%) whereas the RSO contained higher levels of oleic acid (62.2%) and linoleic acid (19.5%), than that of MO (17.4 and 15.8%). The changing fatty acid compositions at different position led to different physical properties. The ω-6/ω-3 ratio of MO and RSO were 0.87 and 2.27, respectively. The balance of ω-6/ω-3 ratio is also an important determinant in decreasing the risk for coronary heart disease. MO contained higher amount of total tocopherols (38.32 mg/100g) but lower amount of total sterols (606.32 mg/100g) than that of RSO (631.98 mg/100g and 25.57 mg/100g). The oxidative stability determined by Rancimat test of MO (PF, 1.57) was higher compared with RSO (PF, 1.0).

Key words: Mustard oil, Rapeseed oil, Fatty acids, Sterols, Tocopherols, Oxidative stability

Introduction

Lipids (oils and fats), apart from providing nutrition, are an integral part of human diets throughout the world, which are products from plants and animals kingdom with great variation in the composition of fatty acids that are of importance for both the chemical and the food industries. Lipids are known to play functional roles in contributing to the palatability of processed foods which provide human nutrition. Actually, consumers tend to prefer oil which is available in their region. In Bangladesh, commonly used edible oils are soybean and mustard oil (MO). In India and Bangladesh, MO is collected from Mustard seeds by traditional way.

MO makes up about 24-40% of the mustard seeds, which is characterized by the presence of higher level of erucic acid and it has the lowest saturated fatty acids content among all the edible vegetable oils. MO has a strong smell, pungent odor

(allyl isothiocyanate), hot and nutty taste. Bangladeshi consumers like such strong smell, hot nutty taste, pungent and sulfurous odor. MO contains fatty acids like erucic, oleic, linoleic and α-linolenic acid. This oil contains a little less than 60% of monounsaturated fatty acids out of which 42% erucic acid and 12% oleic acid. It contains 21% polyunsaturates (6% ω-3 α-linolenic acid and 15% ω-6 linolenic acid) and it has 12% saturated fats (USDA National Nutrient Database). It contains the pungent Allyl isothiocyanate and has about 60% monounsaturated fatty acids of which 42% erucic acid and 12% oleic acid, it has 21% polyunsaturates of which 6% is the omega-3 alpha-linolenic acid and 15% omega-6 linoleic acid and it has 12% saturated fats (USDA National Nutrient Database). MO may provide a protective effect in patients with acute myocardial infarction possibly due to the presence of α-linolenic (Singh et al., 1997). However, higher levels of erucic acid are unsuitable for human consumption as food purposes (Kramer et al., 1982). Generally, erucic acid enriched oil is useful for the polymer industry and is valuable raw material for manufacture of industrial products such as plasticizers, detergents, surfactants, polyesters and coatings whereas oils low in erucic acid are recommended for food purposes because oils high in erucic acid may cause an accumulation of

triacylglycerol in the hearts of animals (Beare-Rogers et al., 1971). Higher consumption of erucic acid may increase the concentration of adrenal cholesterol causing fibrotic changes in myocardium, liver weight and cholesterol (Aaes-Jorgensen, 1972; Beare-Rogers et al., 1972). High levels of erucic acid is not suitable for human food in European Union and other developed countries since erucic acid showed serious pathological changes in the heart and skeletal in animals (Food Standards Australia New Zealand, 2003).

Rapeseed is the third leading source of edible oil in the world. Rapeseed oil (RSO) is the most useful of all cooking oils and it contains a significant amount of n-3 and n-6 fatty acids. RSO contains mostly of the fatty acid such as oleic, linoleic, linolenic, palmitic and strearic acid (Gunstone, et al., 1994; Hui, 1996). The oil content usually makes up about 40-60% of rapeseed. RSO consists 95% of triacylglycerols (TAG) and 5% non-triacylglycerols, known as minor components like free fatty acids, mono- and diacylglycerols, phospholipids, tocopherols, tocotrienols, flavonoids, other phenolic compounds, pigments (chlorophylls), sterols etc (Shahidi and Shukla, 1996).

Every vegetable oil has its own stability against oxidation depending on the fatty acid compositions and the content of antioxidants which it contains (Nogala-Kalucka et al., 2005; Kamal-Eldin, 2006; Przybylski and Eskin, 2006). Figure 1 shows the structure of linoleic (polyunsaturated fatty acid) and erucic acid.
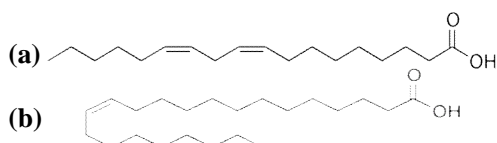

Figure 1. (a) Chemical structure of polyunsaturated fatty acid (linoleic acid); (b) erucic acid (Wikipedia, 2012).

Generally, polyunsaturated fatty acids are responsible for oxidation and off-flavours. However, antioxidant compounds present in oil which is important in the prevention and treatment of diseases such as heart disease, autism, cancer, stroke, diabetes, Alzheimer's dementia, Parkinson's disease, arthritis and muscular degeneration (Manna et al., 2002). It also contributes to lowering serum cholesterol levels in human body (Moreau, 2004) also to the oxidative stability and shelf-life of oil (Przybylski and Eskin, 2006).

The main objectives of this research work were to study the positional fatty acids composition,

retention of sterols and tocopherols, and the oxidative stability of MO and RSO.

Materials and Methods
Oils and chemicals

Mustard oil (MO) was supplied from Agricultural Marketing Co. Ltd. (Dhaka, Bangladesh). Rapeseed oil (RSO) was from AAK (Aarhus Karlshamn AB, Malmö, Sweden). The heptadecanoic acid, acetic acid and pancreatic lipase from porcine pancreas (Type II) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Standard of sterols 5α-cholestane was obtained from Steraloids (Newport, RI, USA), reference samples of tocopherols were obtained from Merck (Darmstadt, Germany) and Tri-Sil reagent was from Pierce Chemical Co. (Rockford, IL, USA). The standard mixture of fatty acid methyl esters (FAME) F-07 was from Larodan Fine Chemicals AB (Malmö, Sweden). All other chemicals and solvents were from VWR (Stockholm, Sweden), unless otherwise stated.

Hydrolysis by pancreatic lipase for positional fatty acid composition

The MO and RSO (8 mg) were taken in a test tube. Eight mL of Tris-HCl buffer (pH 8.0), 2 mL of 0.05% bile salt in distilled deionized water (w/v), 0.8 mL of 2.2% $CaCl_2$ in distilled deionized water (w/v) and 20 mg of pancreatic lipase (porcine pancreatic lipase, crude type II) were mixed for hydrolysis and vortexed for 30 sec. The mixture was incubated in a water bath at 40°C for 3 min, 1 mL of diethyl ether was added. Diethyl ether was evaporated under nitrogen gas. The hydrolytic products were separated on thin-layer chromatography (TLC) plate (silica gel 60, 20x20 cm, 0.25 mm thickness, Merck, Eurolab AB, Stockholm, Sweden) by developing solvent of hexane/diethyl ether/acetic acid (50/50/1, v/v/v). The band of monoacylglycerol was scrapped off for methylation and analyzed by GC. After that, the percentage of fatty acid at sn-1,3 position was calculated by the following formula : Sn-1,3 (%) = (3 T – sn-2)/2 where T is the total fatty acid contents of MO and RSO, respectively. All analyses were conducted in triplicate and GC analysis was done as described above.

Fatty acid composition

The triacylglycerol (TAG) fraction in the MO and RSO were separated by TLC plate (silica gel 60, 20x20 cm, 0.25 mm thickness, Merck, Eurolab AB, Stockholm, Sweden) developed with hexane/diethyl ether/acetic acid (85/15/1, v/v/v). The visualized band corresponding to TAG

molecule was scraped off into a screw-capped tube and dissolved in 0.5 mL hexane. 3 mL 0.01 M NaOH in dry methanol was added into the tube. Fifty μL heptadecanoic acid (C17:0, 1 mg/mL in hexane) as an internal standard was also added in the test tube. The test tube was closed with a stopper and vortexed for proper mixing. The sample was incubated in water bath at 50°C for 30 min under continuous shaking. Two mililiters (50% NaHSO$_4$: 25% NaCl in water) solution were added and cool under running water. Three milliliters water and 1 mL hexane were added and vortexed vigorously. The hexane layer was separated and solvent was evaporated with nitrogen. Gas chromatography (GC, Chrompack CP 9001, Middelburg, The Netherlands), accompanied with auto-injection and flame-ionization detection was used for fatty acid composition analysis. A 50 m X 0.22 mm, 0.25 μm film thickness fused-silica capillary column BPX70 (SGE, Austin, TX, USA) was used for separation. Injector and detector temperatures were 240 and 280°C, respectively. Oven conditions were 160°C increased to 220°C at a rate of 2°C/min and maintained for 5 min. The carrier gas was helium and nitrogen as a make-up gas at a flow rate of 30 mL/min. Fatty acid methyl esters (FAME) were identified by comparison of their retention time with standared FAME. The peak areas were integrated by maestro version 2.4 (Chrompack Middelburg, The Netherlands) and reported as percentage of the total fatty acids (Azardmard-Damirchi and Dutta, 2008). All analyses were conducted in triplicate.

Analysis of sterols by GC

The MO and RSO (20 mg) were taken in a test tube and added 1 mL 2M KOH in ethanol (95%). The tubes was placed in a boiling water bath for 10 min with intermittent shaking and thereafter cooled under running water. Thereafter, 1 mL water, 2 mL hexane containing 20μg 5α-cholestane as internal standard and 200 μL absolute ethanol were added, shaked vigorously and centrifuged. The hexane layer was transferred to a small test tube and evaporated the solvent fully under a stream of nitrogen. For derivatization, 100 μL Tri-Sil reagents was added and dispersed by brief sonication. Then, the sample was incubated at 60ºC for 45 min and dispersed the reagent in the ultrasonic bath. The solvent was evaporated under a stream of nitrogen. The TMS- ether derivates of the sterols were dissolved in 500 μL hexane. About 1 μL sample was injected in splitless mode on GC model 6890 and software ChemStation Rev.

B.02.01 (Agilent Technologies, Wilmington, DE, USA) connected with a GC PAL auto sampler (CTC Analytics AG, Zwingen, Switzerland) and a flame ionization detector. A combination of two fused-silica capillary columns, a DB-5MS (length 15 m, i.d. 0.18 mm and film thickness 0.18 μm) and a DB-35MS (length 10 m, i.di 0.2 mm, and film thickness 0.33 μm) were used which were joined together by a universal press-fit connector. The temperatures of detector and injector were 310 and 260ºC, respectively. The carrier gas was helium and nitrogen was used as make-up gas at flow rates of 0.7 and 30 mL/min, respectively. The initial oven temperature was 60ºC for 1 min and increased to 290ºC at a rate of 50 ºC per min and maintained for 5 min and then increased again by 1ºC per min to 305ºC for 15 min. All analyses were conducted in triplicates and quantification was performed using 5α-cholestane an internal standard.

Analysis of tocopherols by High-Pressure Liquid Chromatography (HPLC)

Quantitative tocopherols content were analyzed by HPLC. The HPLC system consisted of a 510 HPLC pump (Watres, Milford, USA) and Rheodyne Injector with 20 μL loop as well as a fluorescence detector Varian 9070 (Walnut Creek, CA, USA). The detector was set at the wavelength of 294 and 320 nm for excitation and emission, respectively. The column was LiChroCART 250-4 (Merck KGaA, Darmstadt, Germany) packed with Lichrosphere 100 NH$_2$, 5 μm particle size, and coupled to a LiChroCART 4-4 guard column (Merck KGaA, Darmstadt, Germany). Approximately, 10 mg of each sample were dissolved in 1 mL n-heptane and 20 μL were injected manually. The isocratic mobile phase was a mixture of n-heptane: tert-butyl methyl ether. tetrahydrofuran: methanol (79:20:0.98:0.02, v/v/v/v) at the flow rate of 1.0 mL/min. Each tocopherol was quantified using an external standard method and the area of each peak was calculated by an HP 3396A integrator (Hewlett-Packard, Avandale, PA, USA) (Azardmard-Damirchi and Dutta, 2008). All analyses were conducted in triplicate.

Rancimat test for oxidative stability

The induction period, measuring the increase in the volatile by-products released from the oxidizing oil of MO and RSO was determined by the rancimat method (Rancimat 743, Metrohm, Switzerland) at 100ºC with the air flow rate of 25 L/h. The conductivity was measured for estimating the concentration of the degradation products and

longer induction period showed higher oxidative stability. The protection factor (PF), which is calculated by dividing the induction period either of the MO or RSO by that of RSO (induction time, 2.2 h) was obtained as the relative activity (Schwarz and Ernst, 1996).

Results and Discussion
Total and positional fatty acid compositions

The total and positional fatty acid compositions of MO and RSO are presented in Table 1.

The MO and RSO contained total unsaturated fatty acids 94.2 and 92.3% as well as total saturated fatty acids 5.8 and 7.7%, respectively. The MO contained a slightly higher amount of total unsaturated fatty acid (94.2%) than RSO (92.3%). The major fatty acids of MO were erucic (22:1), linolenic (18:3, ω-3), oleic (18:1) and linoleic acid (18:2, ω-6) which composed of 42.8, 18.2, 17.4 and 15.8%, respectively. On the other hand, the RSO contained oleic (18:1), linoleic (18:2, ω-6) and linolenic acid (18:3, ω-3) which composed of 62.2, 19.5 and 8.6%, respectively. The MO contained higher levels of erucic acid (42.8%) and linolenic acid (18.2%), respectively as compared with RSO (0.4 and 8.6%, respectively) whereas the RSO contained higher levels of oleic acid (62.2%) and linoleic acid (19.5%), respectively than that of MO (17.4 and 15.8%, respectively). Almost similar

levels of palmitic acid (16:0) ranged from 4.1 to 4.9% were found in MO and RSO.

The RSO contained higher levels of oleic acid (18:1, 54.0%) at sn-2 position than that of MO (18:1, 35.3%), whereas at sn-2 position, the MO contained higher levels of linoleic acid (18:2, 39.4%) and linolenic acid (18:3, 22.7%) than that of RSO (18:2, 32.4% and 18:3, 13.5%, respectively). The presence of higher amount of unsaturated fatty acids at sn-2 position is important nutritionally, because it is easily converted during digestion and absorbed in the body (Quinlan and Moore, 1993).

The fatty acid contents at sn-1,3 position were erucic (22:1, 63.5%), linolenic (18:3, 15.9%), oleic (18:1, 8.5%), palmitic (16:0, 5.8%) and linoleic (18:2, 4.0%), respectively for MO. In case of RSO, the fatty acid contents at sn-1,3 position were oleic (18:1, 66.3%), linoleic (18:2, 13.1%), palmitic (16:0, 7.3%) and linolenic (18:3, 6.2%), respectively. Such changes of fatty acid compositions at different position lead to different physical properties. The ω-6/ω-3 ratio (n-6/n-3 ratio) of MO and RSO were 0.87 and 2.27, respectively. Healthy ratios of ω-6:ω-3 range from 1:1 to 1:4. An individual needs more ω-3 than ω-6 (Tribole, 2007; Lands, 2005). The balance of ω-6/ω-3 ratio is also an important determinant in decreasing the risk for coronary heart disease both in the primary and secondary prevention of coronary heart disease (Simopoulos, 2002).

Table 1. Positional fatty acids composition (% ±SD) of mustard oil (MO) and rapeseed oil (RSO). All analytical results are presented as mean and standard deviation of triplicate measurement (n = 3).

Fatty acids	MO			RSO		
	sn-1,3	sn-2	Total	sn-1,3	sn-2	Total
16:0	5.8±0.1	0.8±0.05	4.1±0.1	7.3±0.12	0.1±0.05	4.9±0.1
16:1	nd[a]	nd	nd	0.3±0.0	nd	0.2±0.0
18:0	2.3±0.3	0.4±0.05	1.7±0.2	2.7±0.07	nd	1.8±0.05
18:1	8.5±0.2	35.3±0.3	17.4±0.2	66.3±0.1	54.0±0.5	62.2±0.1
18:2 (ω-6)	4.0±0.1	39.4±0.1	15.8±0.1	13.1±0.05	32.4±0.2	19.5±0.1
18:3 (ω-3)	15.9±0.2	22.7±0.2	18.2±0.15	6.2±0.02	13.5±0.2	8.6±0.05
20:0	nd	nd	nd	0.9±0.07	nd	0.6±0.05
20:1	nd	nd	nd	2.1±0.15	nd	1.4±0.1
22:0	nd	nd	nd	0.5±0.2	nd	0.3±0.05
22:1	63.5±0.4	1.4±0.1	42.8±0.3	0.6±0.15	nd	0.4±0.1
24:0	nd	nd	nd	0.2±0.2	nd	0.1±0.05
∑SFA[b]	8.1	1.2	5.8	11.6	0.1	7.7
∑UFA[c]	91.9	98.8	94.2	88.4	99.9	92.3
ω-6/ω-3 ratio			0.87			2.27
Oxidative stability[d]			1.57			1.0

a nd, not detected

b Total sum of saturated fatty acid

c Total sum of unsaturated fatty acid

d Values expressed as protection factor (PF). PF was calculated by dividing the induction period either of the MO or RSO by that of RSO (induction time, 2.2 h)

Table 2. Sterols and tocopherols content (mg/100g±SD) of mustard oil (MO) and rapeseed oil (RSO). All analytical results are presented as mean and standard deviation of triplicate measurement (n = 3).

Sterols and Tocopherols	MO	RSO
Cholesterol	2.03±0.6	7.01±0.31
Brassicasterol	72.11±0.9	59.24±1.66
Campesterol	203.60±2.31	238.86±3.39
Stigmasterol	3.27±0.25	nd
Sitosterol	307.71±4.12	323.90±5.90
Δ5-avenasterol	17.60±.22	2.97±0.31
Total	606.32	631.98
α-tocopherol	5.15±0.28	3.40±0.3
β-tocopherol	0.71±0.15	nd
γ-tocopherol	30.46±0.54	19.44±0.78
δ-tocopherol	2.0±0.29	2.73±0.2
Total	38.32	25.57

Sterols and tocopherols content of mustard oil (MO) and rapeseed oil (RSO)

The distribution of sterols and tocopherols for MO and RSO were assessed. The contents of sterols and tocopherols (mg/100g) of MO and RSO are presented in Table 2.

The contents of cholesterol, brassicasterol campesterol, stigmasterol, sitosterol and Δ5-avenasterol were 2.03, 72.11, 203.6, 3.27, 307.71 and 17.6 mg/100g, respectively) in MO. The RSO contained higher amount of sterols (631.98 mg/100g) than that of MO (606.32 mg/100g). The RSO contained cholesterol (7.01 mg/100g), brassicasterol (59.24 mg/100g), campesterol (238.86 mg/100g), sitosterol (323.90 mg/100g) and Δ5-avenasterol (2.97 mg/100g), respectively. The major sterol in RSO was sitosterol followed by campesterol, brassicasterol, cholesterol, and Δ5-avenasterol. The contents of cholesterol, campesterol and sitosterol (7.01, 238.86 and 323.9 mg/100g, respectively) in RSO were higher than that of MO (2.03, 203.6 and 307.71 mg/100g, respectively). On the other hand, the content of brassicasterol and Δ5-avenasterol (72.11 and 17.6 mg/100g, respectively) were higher in MO than that of RSO (59.24 and 2.97 mg/100g, respectively). Vegetable oils are good sources of tocopherols which are the most important natural antioxidants. The MO contained higher amount of total tocopherols content (38.32 mg/100g) than that of RSO (25.57 mg/100g). The RSO contained 25.57 mg/100g of total tocopherols which concur with previously published results by Gunstone et al. (1994). All samples contained higher amounts of γ-tocopherol than those of α- and δ-tocopherol.

Rancimat test

The oxidative stability is an important parameter in ascertaining the quality of oils and fats, as it gives a good estimation of their susceptibility to oxidative degradation (Aparicio et al., 1999). The results of rancimat test are presented in Table 2. A higher protection factor (1.57) for MO was observed compared to that of RSO (1.0). Generally, higher protection factor suggests stronger oxidative stability. The difference in protection factor of MO was mainly due to different levels of total tocopherols content.

Conclusions

Mustard oil is very popular to the Bangladeshi people due to its strong smell, hot nutty taste, pungent and sulfurous odor. This oil contains higher levels of erucic acid which is not suitable for human consumption as food purposes but it has higher oxidative stability (due to its high content of antioxidants) compared to RSO. Oxidative stability is an important criterion in ascertaining the quality of oils and fats. The balance of ω-6/ω-3 ratio (1:1 to 1:4) is also an important determinant in decreasing the risk for coronary heart disease which was determined in a good range in the case of MO (0.87). Future works are to be designed in our laboratory for the extraction of erucic acid from MO to make this valuable and nutritious vegetable oil more useful as a food product for human consumption.

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Relationship between storage protein banding pattern and growth type of some bread wheat varieties

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Abstract

Seed storage proteins are one of the most important food resources that play important role on bread making quality, in this respect distinguishing the genetic diversity was crucial in breeding aims. In order to study genetic diversity in 20 wheat commercial varieties with different growth type, we analysed the banding patterns of gliadin proteins by SDS-PAGE technique. Our results showed 11 different bands that were polymorphic in 20 studding varieties. Cluster analysis classified these genotypes into 3 groups. These differentiated groups had different growth types; also cluster analysis based on Jaccard similarity coefficient showed that some lines had higher similarities than the others. Principle coordinate analysis showed that 80% of total variance was determined by the first 3 components and cophenetic correlation coefficient equal to 0.75 was resulted and this confirmed the significant relation between gliadins and growth type. Existence of relation between growth type and gliadin proteins could help breeders in variety discrimination and selection perspectives.

Key words: Bread Wheat, Gliadins, Growth type, SDS-PAGE

Introduction

Almost, quality of crop production was the highest emphatic target in any modern breeding program. In cereals bread making quality depend on quality of storage protein and gluten subunits (Dilmer, 1965). Gluten that was the main storage protein composed of gliadin and glutenin (Carillo et al., 1990). Between storage proteins, gliadins, because of high level of genetic diversity, easy extraction and easy analysis by electrophoresis attracted many concerns as biochemical markers. These proteins were used in variety identification and biotypes discrimination within wheat genotypes (Cook, 1992; Kenzewic et al., 1998). These subunits with molecular weight of 28-70 k.Da, consisted about 40 percent of seed storage proteins (Payne et al., 1987). Gliadins constructed from monomer polypeptides and in lower pH in electrophoretic field divided into 4 groups' including α, β, γ and ω (Bushuk and Zilman, 1978; Scofield, 1994). The ω gliadins and the γ gliadins are controlled by genes on the short arms of group

1 chromosomes and α gliadins and the most of β gliadins encoded by genes on the short arms of group 6 chromosomes (Payne et al., 1987).

In order to evaluate genetic diversity the study of biochemical and molecular markers witch are less affected by environmental factors, is more important than morphological traits (Plaschke Röder et al., 1998). Seed storage proteins are the result of expression of genome and contain extensive genetic variation in wheat landraces. Thus, they are taken as good criteria for genetic diversity studies (Porceddue et al., 1998). The most important part of these proteins was polymeric glutenin subunits and monomeric gliadins. Especially, glutenin and gliadin components are important in quality determination as viscoelastic properties of bread wheat flour (Payne & Lawrence, 1983). Bahraii (2004) in studding 43 bread wheat lines found totally of 58 gliadin subunits and deduced that these subunits can used in variety discrimination. Also Farsad (2002) in studding 61 local landraces and 16 breeding lines showed that the gliadin subunits were related with quality characters and these subunits were polymorphic. Metakowsky and Baboev (1992) in 60 *Triticum boeticum* accessions reported that there were 50 different polymorphic bands in this protein studding. Metakowsky and sozinov (1987) showed that storage protein gene blocks had high polymorphism and can be useful as bio- markers.

Growth type in wheat is governed by three major factors such as vernalization requirement, photoperiod sensitivity and narrow-sense earliness. Generally wheat cultivars are divided into two types (winter and spring growth habit) depending on their need for cold temperature to initiate heading. Researchers established the importance of several loci on the 2B chromosome governing heading time in wheat. Other loci that had significant effect on heading time were located on chromosomes 2B, 5B, 1A, 7A and 5A (Kato et al., 1999; Shindo et al., 2003).

In the presence of significant relationship between biochemical marker and any quantitative trait the selection attempts could be facilitated and therefore in this study the presence of this relation was investigated.

Materials and Methods

In this study 20 bread wheat genotypes with different growth types were selected, these 20 genotypes consisted of 10 improved lines and 10 varieties (Table 1). Growth type of studding genotypes were carried out after planting genotypes in greenhouse and the assessment of days to heading traits. In this respect, winter habit wheat is consistent in tillering stages and was not transmitted to booting stage. Extraction of gliadins was based on the method of Singh et al. (1991) and thereafter extracted protein was fractionated by one-dimensional (sodium dodecyl sulfate polyacrylamide gel electrophoresis) SDS-PAGE (12%). Identification of the subunits was performed as described by Payne et al. (1981). Subsequently, cluster analysis was made with NTSYS software after gel scoring (Rohlf, 2000). Gliadin band identification was performed using the Marquis Cultivar. The cluster analysis, correlation coefficient and regression analysis were made using SPSS software.

Table1. Studding lines and Variety name list.

Studding lines	Growth type	Studding Variety	Growth type
1	Winter	Pishgam	Winter
2	Winter	Bezostaya	Winter
3	Winter	Darya	Spring
4	Winter	Saysons	Spring
5	Winter	Koohdasht	Spring
6	Winter	Kaskogene	Winter
7	Facultative	N80-19	spring
8	Winter	Arta	Spring
6.2	Winter	Gaspard	Winter
10	Spring	Shiroodi	Spring

Data Analysis

The data obtained from SDS-PAGE was scored for the presence (1) or absence (0) of the bands and entered in a binary data matrix. Based on the results of electrophoresis band spectra, similarity index was calculated for all possible pair of electrophoregrams. The similarity matrix thus generated was converted to a dissimilarity matrix and used to construct the dendrogram by the unweighted pair group average method (UPGMA). Cluster analysis was applied with Jaccard Similarity coefficient and UPGMA method. Also Principle coordinate analysis according to NEI coefficient was accomplished and for these analyses POPGENE and NTSYS statistical software was used.

Results and discussion

Protein electrophoresis showed 11 clear polymorphic gliadin bands in each studding line (Figure 1). Researchers reported different number of gliadin bands that existed in wheat genotypes. Masoodineghad et al. (1994) reported 20-30 bands, Sepahvand and Vejdani (1995) reported 13-31 bands and Bashuk and Zillman (1978) reported 20-25 bands. Although in each genotype compared to another gliadin bands were polymorphic it resulted in a total up to 132 bands.

Cluster analysis divided the genotypes into 3 groups, first one encompassed lines numbered 1, 2, 7, Bezostaya, Gaspard and Kasgogen that were winter and facultative habited. Second group included line number 3, Saysons, lines numbered 4, 6, 6.2, 5, 8, 10, Pishgam, Darya and Koohdasht genotypes. These genotypes were facultative or moderately spring habited. Third group there were 3 genotypes including: N.80.19, Arta and Shiroodi with spring habit (Figure 2).

The results demonstrated that gliadin electrophoresis could differentiate studding genotypes in respect of their growth habit. Autran and Galterio (1989) in studding 95 durum wheat genotypes using gliadin and total reduced protein electrophoresis concluded that some significant correlation between electerophoretic subunits and agronomic traits existed. Similarly, in the reports of other researchers, a significant correlation between gliadin polymorphism and some agronomic traits such as growth type was considerable (Lee and Kaltsikes, 1973; Cox et al., 1985; Berezovskaya et al., 2005). The study of gliadin subunits could be a key role in variety identification and purity (Branlard et al., 2001; Sozinov, 2001).

On the basis of Jacard similarity index, the most resembling lines were 1, 2 and 6, 6.2 with

approximately index equal to 1 and the least resembling lines with 0.18 index were Darya and Gaspard varieties. Additionally correlation coefficient between cophenetic matrix and primary similarity matrix using mantel test was r = 0.76 with t = 9.9 that was significant (p≤0.01). Rolf (1987) proposed that a correlation between 0.7 and 0.9 had a good fitting. Romesberg (1990) concluded that a correlation coefficient higher than 0.75 represented low deviations between cophenetic matrix and distance matrix and cluster analysis in these circumstances had a high performance (Figure 2). Additionally, spearman correlation coefficient showed some significant correlation between growth type and gliadin banding pattern for example some gliadin band the significant correlation was 0.49, 0.6 and -0.8. These findings confirmed that gliadins could be used as a marker for growth type.

Heterozygotic index between studding genotypes and degree of gene determination on the basis of Nei index (Nei, 1972) was calculated and Ht = 0.34 and Gst =1.00 (Table 2). Our results expressed the exceeding polymorphism of gliadins. Principle coordinate analysis showed that three first component were determined approximately 80 % of total variance that confirmed the cluster analysis grouping too.
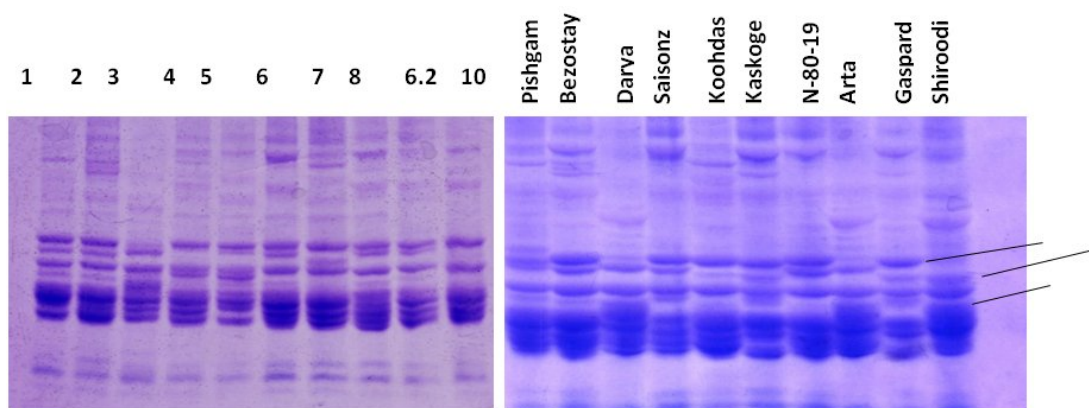

Figure 1. Gliadin electerophoreisis of 20 studding wheat genotypes (the lines showed some significant gliadin bands).

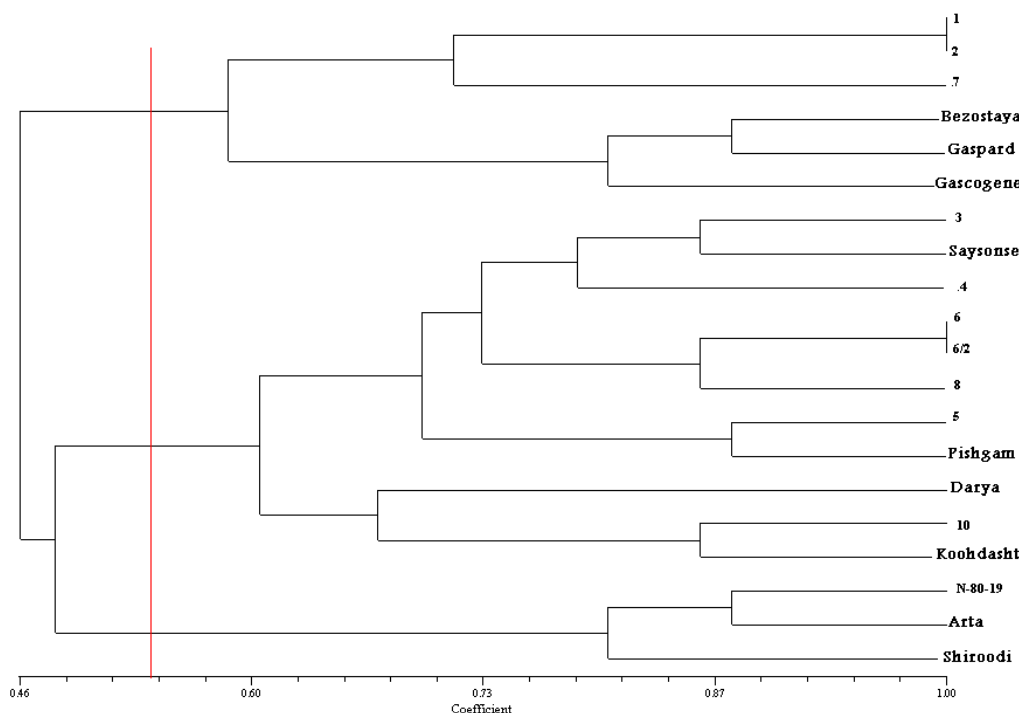Figure 2. Cluster analysis base on gliadin banding pattern, Jaccard coefficient and UPGMA method.

Table 2. Similarity matrix of studding genotypes.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	1.00																			
2	1.00	1.00																		
3	0.50	0.50	1.00																	
4	0.71	0.71	0.71	1.00																
5	0.63	0.63	0.63	0.63	1.00															
6	0.50	0.50	0.71	0.71	0.63	1.00														
7	0.71	0.71	0.50	0.71	0.44	0.71	1.00													
8	0.44	0.44	0.63	0.63	0.56	0.86	0.63	1.00												
6/2	0.50	0.50	0.71	0.71	0.63	1.00	0.71	0.86	1.00											
10	0.50	0.50	0.50	0.71	0.44	0.71	0.71	0.63	0.71	1.00										
Pishgam	0.56	0.56	0.75	0.75	0.88	0.75	0.56	0.67	0.75	0.56	1.00									
Bezostaya	0.75	0.75	0.40	0.56	0.67	0.40	0.56	0.50	0.40	0.40	0.60	1.00								
Darya	0.33	0.33	0.71	0.50	0.44	0.71	0.50	0.63	0.71	0.71	0.56	0.27	1.00							
Saisons	0.63	0.63	0.86	0.86	0.75	0.86	0.63	0.75	0.86	0.63	0.88	0.50	0.63	1.00						
Koohdasht	0.44	0.44	0.44	0.63	0.56	0.63	0.63	0.56	0.63	0.86	0.67	0.50	0.63	0.56	1.00					
Kaskogene	0.50	0.50	0.20	0.33	0.44	0.33	0.50	0.44	0.33	0.33	0.40	0.75	0.20	0.30	0.44	1.00				
N-80-19	0.40	0.40	0.40	0.40	0.67	0.56	0.40	0.67	0.56	0.56	0.60	0.60	0.56	0.50	0.67	0.56	1.00			
Arta	0.30	0.30	0.44	0.30	0.56	0.44	0.30	0.56	0.44	0.44	0.50	0.50	0.63	0.40	0.56	0.44	0.88	1.00		
Gaspard	0.63	0.63	0.30	0.44	0.56	0.30	0.44	0.40	0.30	0.30	0.50	0.88	0.18	0.40	0.40	0.86	0.50	0.40	1.00	
Shiroodi	0.33	0.33	0.33	0.33	0.44	0.33	0.33	0.44	0.33	0.50	0.40	0.56	0.50	0.30	0.63	0.50	0.75	0.86	0.44	1.00

Conclusion

Gliadin proteins were known as quality markers in bread making breeding aims, based on our results some significant relationships between these protein subunits and agronomic trait specially growth type was shown. Therefore, it can be concluded that these protein markers will be very useful in variety identification and growth type. Also chromosomal region that was controlled the above mentioned subunits could be studied as mapping strategies.

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NUTRITION AND FOOD SCIENCE

Contamination levels of selected organochlorine and organophosphorous pesticides in Ghanaian fruits and vegetables

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Abstract

A study was conducted to obtain systematic monitoring data on the contamination levels of selected organochlorine and organophosphorous pesticide residues in fruits and vegetables sold on Ghanaian markets. A total of 309 samples of fruits and vegetables were purchased from the main urban markets and supermarkets in Greater Accra through the months of July, 2009 to May, 2010. The analysis was carried out on GC-ECD employing multi residue analytical technique. The obtained results showed the predominance of methoxychlor in most of the analyzed samples. The detected concentrations of it in pineapple, lettuce, cabbage, cucumber and onion exceeded the European Commission Maximum Residue limits (EC MRLs), as did the concentrations of lindane in papaya, pineapple, cabbage and onion as well as dieldrin in papaya, banana, pineapple and cabbage. Residues of endrin in lettuce and carrot were higher than the EC MRL, as was chlorpyrifos in pineapple. Based on the observations made in these studies, it is proposed that more extensive investigations covering all foodstuffs in Ghana be carried out so as to generate data for policy making, development of consumer information laws and curtailment of the use of some of these pesticides.

Key words: Fruits, Maximum residue limits, Organochlorine, Organophosphorous, Vegetables

Introduction

Pesticides have become widespread pollutants in the environment and now represent a global contamination problem. Hazards associated with these pollutants are their persistence in the environment, their bioaccumulation potential in the tissues of animals and humans through the food chain, and their toxic properties for humans and wildlife (Fu et al., 2003).

These pesticides are widely used on fruits and vegetables because of their susceptibility to insect and diseases. They have been widely used throughout the world since the middle of the last century for their various benefits. Pesticides have been applied in agriculture and animal production to eliminate pests. In this way, to increase both animals and crops outputs, improve quality of products, and decrease the incidence of illnesses propagated by insects (Bempah and Donkor, 2011).

It is therefore not surprising that residues of chlorinated pesticide (OCP) in food have given rise to major concerns. This has reflected in the large number of reports in the literature on this subject (Saeed et al., 2001; Baird and Cann, 2005; Bempah et al., 2011). Moreover, the chronic effects of such exposure levels from food intake are mostly unknown but there is growing evidence of carcinogenicity and genotoxicity as well as endocrine disruption capacity (Miller and Sharpe, 1998) being attributed to the ingestion of or exposure to pesticides. Despite the fact that the use of certain organochlorine pesticides in agriculture is

prohibited in many countries, these compounds have been detected in the environment worldwide due to its persistent nature (Rejendran and Subramanian, 1999).

In an effort to substitute these persistent organochlorine pesticides, agricultural sectors have shifted towards organophosphate pesticides. However, organophosphate pesticides are generally much more toxic to vertebrates compared to other classes of insecticides even though they rapidly degrade in the environment (Chambers et al., 2001). The usage of these pesticides has brought about great concern in the scientific community on the possible toxic effects of these pesticide contaminations to both aquatic flora and fauna as well as to humans (Jorgenson, 2001).

In Ghana, fruits and vegetables production are on the increase to meet the balanced diet requirement for humans, and for better health. Accordingly, everybody is encouraged to consume more fruits and vegetables as they are essential source of vitamins, fiber etc. Therefore, contamination of fruits and vegetables poses a serious health risk to the public. The monitoring of foodstuffs quality is under the responsibility of the Food and Drugs Board (FDB), Ghana. However, pesticide contaminations in food are not documented in the yearly environmental reports and information on pesticide contaminations is generally lacking (Bempah and Donkor, 2011).

The impacts of pesticide contaminations in food have been well studied in North America, Japan and many parts of Europe (Yamaguchi et al., 2003; Gonzalo et al., 2006; Rosa et al., 2008). In contrast, there is very little data on the levels of pesticide residues in developing countries (Albert, 1996). Therefore, monitoring data from developing countries is an important source of information portraying the state of environment in these countries as well as reflecting the effectiveness of environmental policies. To protect consumer's health, many countries have established legal directives to control levels of pesticides in food, through maximum residue levels (FAO/WHO, 2004).

In contrast such legal legislation does not exist in most developing countries, like Ghana so as to minimize the exposure of the consumer to harmful or unnecessary intake of pesticides, to ensure the proper use of pesticides in terms of granted authorization and registration (application rate and pre-harvested intervals) and to permit the free circulation of pesticide treated products, as long as they comply with the fixed MRLs. This may be due to lack of financial support for scientific research, environmental policy and regulations for control and monitoring in the environment. In countries where they exist, the agencies responsible lack the required capacity to ensure compliance and enforcement of regulations (Tchounwou et al., 2002; Bozongo et al., 2004; Bhanti and Taneja, 2007, Osman et al., 2010).

The objective of this study was to obtain systematic monitoring data on the contamination levels of selected organochlorine and organophosphate pesticide residues in fruits and vegetables sold on Ghanaian markets. The selection of these pesticides was based on previous usage except for the organophosphate insecticides. This paper reports the contamination levels of these pollutants in fruits and vegetables sold on the Ghanaian markets.

Materials and methods
Study area

The study area is selected markets in the Greater Accra region of Ghana. These locations are well known for the sale of vegetables and fruits and are the recipients for the fruits and vegetables produce from the urban and rural areas of Ghana.

Sample collection

A total of 309 samples of fruits and vegetables were purchased from the main urban markets and supermarkets in Greater Accra through the months of July, 2009 to May, 2010. The fruit samples used in this study included papaya, water melon, banana, mango, pear and pineapple, while the vegetable samples included tomato, lettuce, cabbage, carrot, onion and cucumber (Table 1). The sample size was at least 1kg for small and medium sized of fresh product. The minimum weight for large sample sizes was 2kg (for example pineapple, cabbage, and water melon), where the unit was generally more than 250g (Codex Alimentarius Commission, 2000).

The samples were sealed and labeled with a unique sample identity and put in an iced chest contained. All samples were transported to pesticide residues laboratory, Ghana Atomic Energy Commission, and were refrigerated (at 5°C). These samples were then extracted and analyzed (within 24 hours from the time of their collection) for the presence of pesticide residues.

Table 1. Number of fruit and vegetables samples analyzed and number of samples with pesticide residue detected.

English name	Scientific name	No. of samples	No. of samples with residues
Fruits			
Papaya	*Carica papaya*	20	13
watermelon	*Citrullus lanatus*	15	8
banana	*Musa sapientum*	34	8
Mango	*Mangifera indica*	25	11
pear	*Pyrus communis*	20	7
pineapple	*Ananas sativus*	25	15
Total		139	62
Vegetables			
Tomato	*Lycopersicon esculentus*	30	15
Lettuce	*Lactuca sativa*	30	14
Cabbage	*Brassica oleracea*	25	17
Carrot	*Daucus carota*	25	13
Onion	*Allium cepa*	30	13
Cucumber	*Cucumis sativus*	30	13
Total		170	85

Sample preparation

Fresh fruit and vegetable samples were thoroughly shredded and homogenized. Approximately 20.0 g of the sample was macerated with 40 ml of ethyl acetate. Sodium hydrogen carbonate 5.0 g and anhydrous sodium sulphate 20.0 g were added to remove moisture and further macerated for 3 minutes using the ultra-turax macerator. The samples were then centrifuged for 5 minutes at 3,000 rpm to obtain the two phases. The supernatant was transferred to a clean graduated cylinder (25 ml) to measure its volume.

Solid-Phase extraction

A solid phase extraction was carried out using SPE column according to Netherlands analytical methods of pesticide residues and foodstuffs with modification (2007). The florisil column (500 mg/8 ml) cartridge was conditioned with 5 ml of a mixture solution of acetone:n-hexane (3:7, v/v) through the column. The sorbent was never allowed to dry during the conditioning and sample loading steps. The extract column was fitted with 20-port vacuum manifold with a receiving flask placed under the column to collect the eluate. Sample loading was performed under vacuum at flow rates of 5 ml min^{-1}. After the passage of the extract, the column was dried by vacuum aspiration under increased vacuum for 30 min. The pesticides were eluted with 10 ml (3, 3, 4 ml) of ethyl acetate, concentrated to 1 ml using a rotary evaporator and then dried by a gentle nitrogen stream. This was dissolved in 1 ml of ethyl acetate; pesticides were then quantified by gas chromatograph equipped with electron capture detector (GC-ECD).

Gas chromatography- electron capture detector (GC-ECD) analysis

Gas chromatograph GC-2010 equipped with ^{63}Ni electron capture detector (ECD) with split/splitless injector that allowed the detection of contaminants even at trace level concentrations (in the lower μg/g range) from the matrix to which other detectors do not respond was employed. The injector and detector temperature were set at 280 ^{o}C and 300°C respectively. A fussed silica ZB-5 (30 m x 0.25 mm, 0.25 μm film thickness) was used in combination with the following oven temperature program: initial temperature 60°C, held for 1 min, ramp at 30°C min^{-1} to 180°C, held for 3 min, ramp at 3°C min^{-1} to 220°C, held for 3 min, ramp at 10°C min^{-1} to 300°C. Nitrogen was used as carrier gas at a flow rate of 1.0 ml min^{-1} and make up gas of 29 ml min^{-1}. The injection volume of the GC was 1.0 μl. The residues detected by the GC analysis were confirmed by the analysis of the extract on two other columns of different polarities. The first column was coated with ZB-1 (methyl polysiloxane) connected to ECD and the second column was coated with ZB-17 (50% phenyl, methyl polysiloxane) and ECD was also used as detector. The conditions used for these columns were the same.

Quality control and quality assurance

Quality control and quality assurance were included in the analytical scheme. The recovery, precision and linearity of studied pesticides were evaluated by adding a working mixture to 20 g of chopped untreated samples; the spiked samples were made to stand for at least 1 hour before the

extraction. Ten replicate samples were extracted and analyzed according to the proposed procedure as described previously. Precision was calculated based on daily repeatability of 10 samples, whereas reproducibility was carried out on 5 different days. Recoveries were calculated for three replicate samples. Percent recoveries in spiked samples ranged 87% - 120%. Accordingly, the sample analysis data were corrected for these recoveries. Detection limit(s) of the method were also assessed based on the lowest concentrations of the residues in each of the matrices that could be reproducibly measured at the operating conditions of the GC; which were 0.001mg/kg. Blank analyses were also carried in order to check any interfering species in the reagents.

Results

Organophosphorus pesticides are widely used in agriculture and animal production for the control of various insects. These compounds have higher acute toxicity than chlorinated pesticides and they have the advantage of being more rapidly degraded in the environment. Organochlorine pesticides, which over a decade ago were being used in Ghana, are highly persistent. Most of them have been banned, yet their residues still appear as pollutants in food as well as in the environment. Residue levels of these compounds in fruits and vegetables are listed in Tables 2 and 3.

Levels of pesticide residues found in fruits collected from the various market centers

The identities of all the two groups of pesticide (organochlorine and organophosphorus) residues found in fruits are given in Table 2. Among the various organochlorine pesticides in the present study, lindane is the predominant compound in the fruit samples. The detected levels of it varied greatly. For instance, the minimum value for it was detected in watermelon (0.004mg/kg) and the maximum of 0.133mg/kg was found in pineapple.

Table 2. The detected levels (mg/kg) of pesticide residues in Ghanaian fruits samples.

Pesticide types	Papaya	Watermelon	Banana	Mango	Pear	Pineapple
Organochlorines						
Lindane	0.100*±0.004	0.004±0.002	<LOD[b]	0.010±0.010	0.009±0.003	0.133*±0.014
	(0.092-0.105)[a]	(0.004-0.006)	-	(0.006-0.022)	(0.006-0.012)	(0.121-0.153)
Methoxychlor	0.006±0.002	<LOD	0.008±0.004	0.004±0.001	<LOD	0.031*±0.023
	(0.004-0.012)	-	(0.004-0.012)	(0.004-0.006)	-	(0.007-0.052)
Aldrin	0.004±0.002	<LOD	<LOD	<LOD	0.007±0.003	0.006±0.002
	(0.001-0.008)	-	-	-	(0.003-0.009)	(0.004-0.008)
Dieldrin	0.017*±0.020	<LOD	0.090*±0.103	<LOD	<LOD	0.012*±0.008
	(0.002-0.040)	-	(0.013-0.203)	-	-	(0.007-0.018)
Endrin	<LOD	<LOD	0.006±0.002	<LOD	<LOD	0.004±0.002
	-	-	(0.004-0.012)	-	-	(0.004-0.008)
p,p'-DDE	<LOD	0.004±0.001	<LOD	0.010±0.004	<LOD	<LOD
	-	(0.004-0.008)	-	(0.005-0.011)	-	-
p,p'-DDT	0.012±0.006	0.008±0.004	0.038±0.032	0.020±0.002	<LOD	<LOD
	(0.008-0.014)	(0.006-0.010)	(0.005-0.062)	(0.018-0.021)	-	-
Organophosphorus						
Diazinon	<LOD	<LOD	<LOD	<LOD	<LOD	0.007±0.003
	-	-	-	-	-	(0.001-0.009)
Dimethoate	0.008±0.002	0.004±0.001	<LOD	0.010±0.014	<LOD	0.006±0.002
	(0.002-0.012)	(0.004-0.006)	-	(0.004-0.018)	-	(0.002-0.008)
Pirimiphos-methyl	<LOD	<LOD	<LOD	0.004±0.001	<LOD	0.014±0.012
	-	-	-	(0.002-0.006)	-	(0.008-0.018)
Chlorpyrifos	0.008±0.002	0.003±0.002	0.006±0.002	0.005±0.003	0.017±0.007	0.055*±0.011
	(0.004-0.010)	0.002-0.005	(0.004-0.012)	(0.003-0.007)	(0.012-0.025)	(0.041-0.062)
Profenofos	0.003±0.002	<LOD	<LOD	<LOD	<LOD	<LOD
	(0.001-0.005)	-	-	-	-	-
Malathion	<LOD	<LOD	<LOD	<LOD	<LOD	0.006±0.002
	-	-	-	-	-	(0.002-0.008)

Each value is the mean of five samples with four determinations

a Range in bracket

b LOD= 0.001 mg/kg sample ; Values designated by asterisks are higher than the EC-MRLs for the respective pesticide (see MRLs in Table 4-5)

Minimum values for methoxychlor were detected in mango (0.004mg/kg) and the maximum of 0.031mg/kg was found in pineapple. 0.004mg/kg of aldrin was detected in papaya and the maximum of 0.007mg/kg was found in pear. Minimum value of 0.012mg/kg of dieldrin was found in pineapple and maximum value of 0.090mg/kg was detected in banana whiles 0.004mg/kg of endrin was found in pineapple and maximum of 0.006mg/kg was found in banana.

Moreover, p,p'-DDE was having a minimum value of 0.004mg/kg in watermelon and maximum value of 0.010mg/kg in mango while p,p'-DDT recorded a minimum value (0.008mg/kg) in watermelon and maximum value of 0.038mg/kg was found in banana.

With respect to the organophosphorus pesticides, Table 2 shows that diazinon and malathion were the least predominant pesticide with residues of 0.007mg/kg and 0.006mg/kg in pineapple samples. However, chlorpyrifos was the most dominant pesticide residues. The minimum value of 0.003mg/kg of it was found in watermelon and the maximum value of 0.055mg/kg in pineapple. Following chlorpyrifos is dimethoate, where the minimum value (0.004mg/kg) of it was found in watermelon and maximum value of 0.010mg/kg in mango. Minimum value of pirimifos-methyl (0.004mg/kg) was detected in mango and maximum value of 0.014mg/kg was found in pineapple.

Levels of pesticide residues found in vegetables collected from the various market centers

In regard to the organochlorine pesticides found in vegetable samples, Table 3 indicates that methoxychlor and p,p'-DDT were the most predominant pesticides with maximum residual concentrations of 0.041mg/kg and 0.035mg/kg present in onion samples while minimum residual concentrations of 0.004mg/kg each were also present in tomato and carrot samples, respectively.

Table 3. The detected levels (mg/kg) of pesticide residues in Ghanaian vegetables samples.

Pesticide types	Tomato	Lettuce	Cabbage	Carrot	Cucumber	Onion
Organochlorines						
Lindane	0.008±0.002	0.006±0.002	0.100*±0.004	<LOD[b]	<LOD	0.019*±0.002
	(0.004-0.010)[a]	(0.004-0.006)	(0.095-0.102)	-	-	(0.016-0.020)
Methoxychlor	0.004±0.002	0.023*±0.008	0.023*±0.008	0.008±0.004	0.020*±0.002	0.041*±0.022
	(0.002-0.008)	(0.031-0.022)	(0.031-0.022)	(0.006-0.012)	(0.018-0.021)	(0.025-0.066)
Aldrin	<LOD	0.008±0.004	<LOD	0.010±0.021	<LOD	0.006±0.002
	-	(0.006-0.012)	-	(0.008-0.040)	-	(0.004-0.008)
Dieldrin	0.004±0.008	<LOD	0.035*±0.013	<LOD	0.010±0.004	<LOD
	(0.002-0.040)	-	(0.030-0.052)	-	(0.005-0.013)	-
Endrin	<LOD	0.040*±0.035	0.007±0.003	0.016*±0.008	<LOD	<LOD
	-	(0.080-0.015)	(0.005-0.009)	(0.006-0.032)	-	-
p,p'-DDE	0.013±0.009	0.041±0.022	0.008±0.004	<LOD	<LOD	0.023±0.008
	(0.007-0.015)	(0.025-0.066)	(0.006-0.010)	-	-	(0.016-0.031)
p,p'-DDT	0.012±0.006	0.020±0.002	0.032±0.010	0.004±0.002	0.009±0.003	0.035±0.005
	(0.008-0.014)	(0.018-0.021)	(0.030-0.040)	(0.004-0.008)	(0.005-0.013)	(0.030-0.040)
Organophosphorus						
Diazinon	0.009±0.003	0.004±0.001	0.016±0.005	0.005±0.002	0.009±0.006	0.008±0.004
	(0.003-0.013)	(0.002-0.006)	(0.010-0.019)	(0.003-0.011)	(0.003-0.011)	(0.004-0.010)
Dimethoate	0.013±0.009	0.021±0.013	<LOD	0.020±0.014	<LOD	0.006±0.002
	(0.007-0.019)	(0.018-0.024)	-	(0.018-0.024)	-	(0.002-0.008)
Pirimiphos-methyl	0.017±0.007	<LOD	0.003±0.001	<LOD	0.010±0.007	<LOD
	(0.012-0.025)	-	(0.001-0.006)	-	(0.006-0.021)	-
Chlorpyrifos	0.026±0008	0.011±0.010	0.007±0.003	0.040±0.026	<LOD	0.055±0.011
	(0.018-0.025)	(0.001-0.021)	(0.003-0.009)	(0.038-0.044)	-	(0.041-0.062)
Profenofos	0.010±0.004	<LOD	0.008±0.004	0.012±0.009	0.003±0.001	0.040±0.002
	(0.005-0.011)	-	(0.002-0.010)	(0.010-0.016)	(0.001-0.009)	(0.008-0.044)
Malathion	0.038±0.032	0.003±0.003	0.004±0.001	0.007±0.003	0.010±0.008	<LOD
	(0.005-0.062)	(0.001-0.006)	(0.004-0.008)	(0.005-0.011)	(0.008-0.012)	-

Each value is the mean of five samples with four determinations

a Range in bracket

b = 0.001 mg/kg sample

Values designated by asterisks are higher than the EC-MRLs for the respective pesticide (see MRLs in Table 4-5)

In contrast with the trend exhibited in methoxychlor and p,p'-DDT, lindane recorded a minimum value of 0.006mg/kg in lettuce and maximum value of it (0.010mg/kg) was recorded in cabbage. Unlike lindane, p,p-DDE recorded a maximum value in lettuce (0.041mg/kg) and a minimum value in cabbage (0.008mg/kg). Maximum residual concentrations of aldrin, dieldrin and endrin were detected in carrot, cabbage and lettuce to be 0.010, 0.035 and 0.040mg/kg, respectively while minimum concentration values were detected in onion (0.006mg/kg), tomato (0.004mg/kg) and cabbage (0.007mg/kg), respectively.

In the case of organophosphorus pesticides found in vegetable samples, diazinon is the most predominant pesticide residues found in the analyzed vegetable samples with maximum concentration levels of it (0.016mg/kg) found in cabbage and a minimum value of 0.004mg/kg found in lettuce. Next to diazinon are chlorpyrifos, profenofos and malathion. Chlorpyrifos achieved a maximum value of 0.055mg/kg in onion and a minimum value of 0.007mg/kg in tomato samples analyzed. Corresponding values for profenofos were 0.012mg/kg in carrot and 0.003mg/kg in cucumber samples, respectively. Furthermore, the maximum detected residue of malathion was detected in tomato (0.038mg/kg), and the minimum value was found in lettuce (0.003mg/kg). Maximum concentration values of dimethoate and pirimifos-methyl were detected in lettuce and

tomato to be 0.021mg/kg and 0.017mg/kg respectively whiles minimum concentration values were also detected in onion (0.006mg/kg) and cabbage (0.003mg/kg) samples, respectively.

The data further showed occurrence of some pesticide residues in fruits and vegetables at levels exceeding maximum residue limits (MRLs). Compared with the MRLs established by EC (2006), methoxychlor is most often exceeded MRL values (41.6%), followed by lindane and dieldrin (33.3%), endrin (16.6%) and chlorpyrifos (8.3%) (Tables 4 and 5).

Overall residues were found in 41.4% of fruit samples and 58.9% of vegetable. The reason for this might be that, vegetables are highly sensitive to pest and need for successive applications of pesticides treatments, leaving in consequence higher level of residues that tolerated and protected from pest infestation.

From this work, it can be seen that, lindane is detected in papaya, pineapple, cabbage and onion at levels higher than the MRLs set by EC (2006). Also, methoxychlor was higher in pineapple, lettuce, cabbage, cucumber and onion than the MRL. It can also be seen that dieldrin detected in papaya, banana, pineapple and cabbage were higher than the MRL. Also, endrin in lettuce and carrot as well as chlorpyrifos in pineapple were detected at levels higher than the MRL recorded by EC (Table 4). The results indicated that pesticides should be applied correctly using only the required amounts and following label directions.

Table 4. Maximum residue levels for organochlorine pesticides in the selected fruits and vegetables.

Commodity	Maximum residue levels, MRLs (mg/kg)						
	Gamma-HCH	methoxychlor	aldrin	dieldrin	endrin	p,p'-DDE	p,p'-DDT
Fruits							
Papaya	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Water melon	0.01	0.01	0.03	0.03	0.01	0.05	0.05
Banana	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Mango	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Pear	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Pineapple	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Vegetables							
Tomato	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Lettuce	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Cabbage	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Carrot	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Onion	0.01	0.01	0.01	0.01	0.01	0.05	0.05
Cucumber	0.01	0.01	0.02	0.02	0.01	0.05	0.05

Table 5. Maximum residue limits for organophosphorus pesticides in the selected fruits and vegetables.

Commodity	Maximum residue levels, MRLs (mg/kg)					
	Diazinon	Dimethoate	Pirimiphos-methyl	Chlorpyrifos	Profenfos	Malathion
Fruits						
Papaya	0.02	0.02	0.05	0.50	0.05	-[a]
Water melon	0.02	0.02	0.05	0.05	0.05	-
Banana	0.02	0.02	0.05	3.00	0.05	0.50
Mango	0.02	0.02	0.05	0.05	0.05	-
Pear	0.30	0.02	0.10	0.50	0.05	0.50
Pineapple	0.02	0.02	0.05	0.05	0.05	-
Vegetables						
Tomato	0.50	0.02	0.20	0.50	0.05	3.00
Lettuce	0.02	0.50	0.50	0.05	0.05	3.00
Cabbage	0.02	0.02	0.50	0.50	0.05	-
Carrot	0.20	0.02	0.05	0.10	0.05	0.50
Onion	0.50	0.02	0.10	0.20	0.05	3.00
Cucumber	0.02	0.02	0.10	0.05	0.05	3.00

[a]-No EC MRL

Discussion

The occurrence of selected organochlorine and organophosphate pesticides was studied in the selected urban markets and supermarkets in Greater Accra region of Ghana. The pesticides detected were lindane, methoxychlor, aldrin, dieldrin, endrin, p,p'-DDE, p,p'-DDT, diazinon, dimethoate, pirimiphos-methyl, chlorpyrifos, profenofos and malathion. This study has shown the presence of organochlorine pesticides despite the fact that they have been banned for a considerable amount of time in Ghana. This study therefore suggest the possibility of sporadic use of these pesticides for agriculture or mainly due to the past extensive use of these pesticides for agriculture in Ghana as it has been banned for over a decade ago.

These findings corroborate the findings of Nakata et al. (2002) who found elevated levels of organochlorine pesticides residues in fruits and vegetables collected from Shanghai and Yixing, China. Similarly, in an investigation carried out by Hura (1999), by monitoring organochlorine residues in fruits and vegetables at Eastern Romania, it was concluded that organochlorine pesticides were found in all analyzed samples. A similar research conducted by Mukherjee et al. (2011) in West Bengal, India, to access the level of organochlorine pesticides residues in vegetables revealed that, the concentration of ∑OCPs was ranged between, <0.01– 65.07µg/kg with average of 9.67±2.34µg/kg (wet wt.). The concentration of ∑DDT, ∑HCH, aldrin, dieldrin and heptachlor was 3.49±0.93µg/kg, 2.07±0.53µg/kg, 1.32±0.65µg/kg, 1.36±1.18µg/kg and 1.80±0.4µg/kg (wet wt) respectively

The data therefore show the decreased concentrations of the residues of organophosphorus pesticides, which were detected in some of the analyzed samples of fruits and vegetables under investigation except chlorpyrifos which exceeded MRL in pineapple samples. This might be due to its ability to degrade rapidly in the environment than organochlorine pesticides. Similar results were obtained by Abou-Arab and Abou Donia (2001) who found that samples collected from Egypt contained organophosphorus pesticides particularly, malathion, dimethoate and profenofos at levels ranging from 0.061 to 1.756mg/kg. In 2006, Bai et al. (2006) concluded that the OP pesticide residues were present in fruits and vegetables in Shaanxi area of China.

Conclusion

Contamination of the fruits and vegetables with these pesticide residues poses a significant health risk to the public from consuming contaminated fruits and vegetables. The other foodstuffs are also threatened by the presence of pesticides residues. The presence of pesticide residues is attributed to more quantity and cosmetic quality of these commodities which create over reliance on pesticides. While greater portion of pesticides being consumed in the country are used on fruits and vegetables. This monitoring study is being continued to provide more information on the pesticide contaminations in foodstuffs in Ghana, which will further contribute to the information available on pesticide residues found in the food commodities in Ghana.

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PLANT SCIENCE

Effects of scarification and nutrient mineral concentrations on the *in vitro* germination of *Senna macranthera* (Collad.) H. S. Irwin & Barneby seeds

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Abstract

Senna macranthera is a tree species used in the recovery of degraded ecosystems whose wood is also used in carpentry and for cooking. Extracts of its leaves and seeds have shown potential pharmacological activities. The seeds demonstrate dormancy, which affects seedling production and the commercial propagation of the trees. We evaluated different methods for overcoming seed dormancy and the effects of nutrient media concentrations and light on the *in vitro* germination of *S. macranthera*. Seeds were subjected to chemical scarification with H_2SO_4 or mechanical scarification using sandpaper. Non-scarified seeds were used as control. Seeds were inoculated into test tubes containing MS, MS ½, or WPM medium and kept in growth rooms under a 16L:8D photoperiod or in total darkness. Scarification treatments promoted greater germination percentages than controls under all germination conditions tested. Sixty days after inoculation, the seedlings germinated from scarified seeds had greater root and shoot lengths than those of the controls, regardless of the culture medium used. It can be concluded that the physical methods used for overcoming dormancy are necessary and effective in promoting *in vitro* germination and subsequent seedling growth.

Key words: Dormancy, *In vitro* germination, Scarification, *Senna macranthera*

Abbreviations: MS = Murashige and Skoog medium; MS½ = half concentrations of MS macro and micronutrients; H_2SO_4 = sulphuric acid; WPM = Lloyd & McCown medium; SGI = speed germination index; % G = germination percentage; APL = aerial part length; RPL = root system length

Introduction

Senna macranthera (Collad) H.S. Irwin & Barneby (Fabaceae – Caesalpinoideae) is a tree species commonly found in semi-deciduous altitudinal forests; it can grow up to 8 m in height with a trunk diameter of 20 to 30 cm, the leaves are composed of two pairs of opposite folios (Lorenzi, 1992). This species is widely utilized in both rural and urban landscaping and its wood can be used in carpentry and as firewood.

Species of the genus *Senna* have been found to produce a wide variety of bioactive compounds and more than 350 secondary metabolites are known from species growing in tropical and subtropical regions (Viegas et al., 2006). Extracts of *S. macranthera* bark contain compounds such as rubrofusarin, which shows significant biological activity (Pereira et al., 1995; Mata et al., 2003; Song et al., 2004; El-Halawany et al., 2007), and extracts of the seed endosperm have been shown to have anti-coagulant activity (Pires et al., 2001). Nogueira (2009) reported anti-bacterial, laxative, anti-inflammatory and anti-oxidant activities of ethanol extract fractions of *S. macranthera* leaves and this species demonstrates great economic potential as a source of tannins and galactomannans (Santarém and Aquila, 1995).

S. macranthera is a pioneer species indicated for recuperating degraded ecosystems (Lorenzi, 1992). Its utilization in forest recuperation projects has been limited, however, by a lack of information concerning its biology and ecology or the appropriate techniques for propagation and management (Ranieri et al., 2003).

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In spite of the potential usefulness of *S. macranthera* to humans, its cultivation has been limited by difficulties encountered in obtaining commercial stocks of seedlings due to its low seed germination rate. Seed dormancy has been observed in a large number of forest species, and is considered an adaptive mechanism under natural conditions (Bruno et al., 2001). Seed dormancy is one of the principal problems facing attempts to produce seedlings of native forest species and conserve their germplasm (Oliveira et al., 2003; Dôres, 2007). *S. macranthera* seeds demonstrate dormancy caused by the impermeability of the tegument (Filho et al., 1997; Santarém and Aquila, 1995), which impedes imbibition, but it can be overcome by chemical scarification using sulfuric acid or mechanical abrasion (Santarém and Aquila, 1995; Eschiapati and Perez, 1997).

Tissue culture has been successfully used to propagate and conserve many economically important plants species and maximize large-scale seedling production efforts – thus reducing the need for *in situ* harvesting of natural native forest resources (Bapat et al., 2008)

The present work evaluated the effects of scarification by sulfur acid or mechanical abrasion and exposure to different concentrations of culture media on overcoming dormancy of *S. macranthera* seeds and on their subsequent *in vitro* growth.

Materials and Methods

S. macranthera seeds were acquired through the Clube da Semente (www.clubedasemente.org) and remained stored in paper envelopes at room temperature until used.

The seeds were submitted to the following treatments to overcome dormancy: control (seeds not subjected to any scarification); chemical scarification (immersion in 98% concentrated sulfuric acid for 45 min.); mechanical manual scarification (abrasion on the side of the seed opposite the embryo, using sterilized 100 grit sandpaper).

The scarified and non-scarified seeds were disinfected by immersion in 70% ethyl alcohol for 1 min. and then 2.5% sodium hypochlorite for 10 minutes followed by rinsing in sterile distilled water. After disinfection, the seeds were inoculated into test tubes containing the following sterile culture media: standard MS medium (Murashige and Skooge, 1962); MS½ medium and 3% sucrose; and WPM medium (Lloyd & McCown, 1980) with 2% sucrose. The pHs of the three media types were just to 5.8 ± 0.1 before adding 6.0 g L^{-1} of agar and autoclaving at 121°C and 1.1 kg cm^{-2} for 20 minutes.

The seeds inoculated into the different media were then maintained at 25 ± 2°C under two different illumination regimes: 1) a 16L:8D photoperiod with 42 μmol m^{-2} s^{-1} of PAR furnished by fluorescent daylight-type lamps; 2) total darkness.

The experiments were conducted by testing all possible combinations of culture media, scarification methods, and illumination regimes, with five repetitions each; each trial involved 10 test tubes with one seed each.

Daily observations were made to accompany seed germination and to calculate the speed germination index (SGI) (Maguire, 1962) and germination percentage (%G). Seeds were considered to have germinated if they demonstrated development of their aerial portion. After the germination percentages had stabilized, measurements were made of the aerial part length (APL) and root system length (RPL). The data were submitted to analysis of variance at a 5% probability level and compared using the Duncan and Tukey tests, all run on the STATISITCA program for Windows.

Results and Discussion

S. macranthera seeds germinated well in both presence and absence of light and can therefore be considered photoblastically neutral (Table 1). The methods tested for overcoming dormancy were efficient under both light and dark conditions, always demonstrating greater %G than seen among the controls (Table 1).

Table 1. Percentage germination of *S. macranthera* seeds in different culture media under different illumination conditions.

Percentage germination (%G)						
Light				Dark		
Medium	Sulfur acid	Sandpaper	Control	Sulfuric acid	Sandpaper	Control
MS	68 ab A	58 b A	28 c A	56 b A	80 a A	18 c A
MS ½	38 b B	54 ab A	20 c A	62 a A	46 ab B	18 c A
WP	56 a AB	52 ab A	36 bc A	62 a A	62 a B	30 c A

Values followed by the same lowercase letter on the same line, or the same uppercase letters in the same columns, do not statistically differ at the 5% probability level by the Duncan test.

Table 2. Speed Germination Index of *S. macranthera* seeds in different culture media.

Medium	Speed Germination Index (SGI)		
	Sulfuric acid	Sandpaper	Control
MS	0.059 aA	0.041 bA	0.021 bA
MS ½	0.034 abA	0.046 aA	0.016 bA
WPM	0.055 aA	0.041 aA	0.033 aA

Values followed by the same lowercase letter on the same line, or the same uppercase letters in the same columns, do not statistically differ at the 5% probability level by the Duncan test.

Seeds that had been scarified with sulfuric acid and germinated under light conditions showed greater %G in the MS and WPM media. Mechanically scarified seeds demonstrated no significant differences in %G in any of the growth media, indicating that the different mineral salt concentrations did not greatly affect their germination. According to Eschiapati and Perez (1997), immersion in 98% sulfuric acid for 50 min. to overcome dormancy resulted in the highest germination rates of *S. macranthera* seeds when germinated on humidified filter paper. Chemical scarification of *S. macranthera* seeds with dark incubation resulted in germination percentages that did not statistically differ from the control; mechanical scarification with sandpaper with dark incubation promoted higher %G only in the MS media.

The analyses of the germination responses in the different culture media indicated that under conditions of illumination both mechanical or chemical scarification were efficient in promoting higher %G in relation to the control in the MS and WPM media (Table 1). Under dark conditions, mechanical scarification yielded the highest %G in the MS medium, although the MS ½ and WPM media both gave satisfactory results. Filho et al. (1997) and Santarém and Aquila (1995) examined the germination of *S. macranthera* seeds on filter paper and observed that overcoming dormancy using sandpaper or sulfuric acid for 12 and 15 minutes respectively was sufficient to overcome dormancy. These same methods were found to be deficient in overcoming dormancy in *Senna siamea* seeds, with the greatest %G been seen with diaspores sown onto humidified filter paper (Dutra et al., 2007).

Table 2 shows that the SGI values were not significantly influenced by the mechanical or chemical methods used for overcoming dormancy or the types of culture media used. Scarification with sulfuric acid gave the best results in the MS medium. This same result was observed by Eschiapati and Perez (1997) who scarified seeds by immersion in sulfuric acid for 50 min. and observed 90% germination within 2.17 days on filter paper. The greatest SGI values in *Mimosa caesalpiniaefolia* were also observed under these same conditions (Bruno et al., 2001). MS ½ favored high SGI with both types of scarification, while there was no significant difference between the scarification treatments when compared to the control in WPM medium (Table 2). Santarém and Aquila (1995) observed that, independent of the storage time of *S. macranthera* seeds, mechanical scarification resulted in germination rates above 80% among seeds sown onto filter paper.

When the lengths of the aerial parts of the seedlings were evaluated, the greatest growth was observed to have occurred among plants cultivated in the dark, without significant statistical differences between the two scarification treatments and the control (Table 3 and Figure 1). Both scarification techniques resulted in high APL values when the seeds were inoculated into MS or MS ½ media, but the results of these treatments and the control were inferior to those in the WPM medium.

According to Coelho et al. (2001), techniques that help overcome seed dormancy can result in high APL values due to the acceleration of the imbibition processes and consequent rapid seed germination. The greatest APL values in *Zizyphus joazeiro* (Rhmneae) were observed among seedlings derived from seeds scarified with sulfuric acid (Alves et al., 2006).

Table 3. Lengths of the aerial portions (cm) of *S. macranthera* seedlings grown in different culture media and under different light conditions.

	Length of the aerial portion (APL)					
	Light			Dark		
	Sulfuric acid	Sandpaper	Control	Sulfuric acid	Sandpaper	Control
MS	7.36 b A	6.15 b A	5.91 b A	16.73 a A	16.92 a A	14.07 b A
MS ½	6.82 ac A	7.81 ac A	5.16 b A	17.07 a A	18.73 a A	10.86 b A
WPM	6.23 b A	7.30 b A	5.48 b A	14.17 b A	13.29 b B	17.31 a A

Values followed by the same lowercase letter on the same line, or the same uppercase letters in the same columns, do not statistically differ at the 5% probability level by the Duncan test.

Table 4. Lengths of the radicles (cm) of *S. macranthra* seedlings grown in different culture media and under different light conditions.

Length of the radicle (RPL)						
	Light			Dark		
	Sulfuric acid	Sandpaper	Control	Sulfuric acid	Sandpaper	Control
MS	5.46 ae A	5.17 bcde A	4.94 bde A	6.94 a A	6.61 ac A	6.37 ad A
MS ½	6.45 ac A	6.43 ab A	4.15 bcde A	7.51 a A	5.21 ae B	3.48 be B
WPM	5.59 a A	5.03 a A	4.93 a A	6.02 a A	4.43 a B	4.41 a B

Values followed by the same lowercase letter on the same line, or the same uppercase letters in the same columns, do not statistically differ at the 5% probability level by the Duncan test.

In relation to the radicule length, no significant differences were noted between the different methods of scarification in the three different cultivation media under light conditions (Table 4). The same scarification techniques likewise did not significantly influence RPL values among seeds cultivated in the MS and WPM media (Table 4); in the MS ½, chemical scarification was observed to be more efficient in relation to the control but statistically equivalent to mechanical scarification.

Pterodon pubescens seeds germinated *in vitro* in MS medium showed the greatest APL, RPL, and %G values when their teguments were removed (Coelho et al., 2001), which corroborates our results. Martins and Nakagawa (2008) observed that mechanical scarification did not influence radicule development in *Stryphnodendron adstringens* seeds germinated on paper towels.

Data in the published literature indicates that overcoming seed dormancy by mechanical scarification can compromise the RPL of seedlings as the scarification is performed in the region on the opposite side of the seed from the radicle and the region where the embryo is located thus remains impermeable – which slows water infiltration into the embryo and maintains the physical barrier (the seed coat) to initial root growth (Martins and Nakagawa, 2008). The effectiveness of sulfuric acid in overcoming tegument-imposed dormancy is related to the removal of the cuticle and exposure of the macro-sclereids, thus allowing the uniform imbibition of the seed and its rapid germination – which can result in seedlings having more highly developed aerial and radicle organs (Santarém and Aquila, 1995).

Figure 1. *S. macranthera* seedlings germinated *in vitro*. (a) seedling cultivated in WPM medium in the light from seeds that did not receive any treatment for overcoming dormancy (control), (b) seedlings cultivated in the dark in WPM medium derived from seeds that had been mechanically abraded to overcome dormancy, (c) seedling cultivated under illumination conditions in WPM medium derived from seeds that had been abraded to overcome dormancy, (d) seedling cultivated in the dark in WPM medium derived from seeds that had been immersed in sulfuric acid to overcome dormancy.

Conclusions

Chemical and mechanical scarification were efficient in overcoming dormancy in *S. macranthera* seeds and favored their germination under both light and dark incubation conditions; nutrient mineral concentrations in the culture media influenced germination responses. The greatest germination percentages were observed in MS medium with chemical scarification under light incubation conditions, and with mechanical scarification and incubation in the dark. The different scarification treatments did not influence the SGI values in relation to the different culture media utilized. The greatest SGI value in MS medium was obtained with chemical scarification. The length of the aerial part of the seedlings was principally influenced by germination in the dark, and scarification of the seeds with sulfuric acid or sandpaper favored the development of the aerial portions of the seedlings cultivated in both the MS and MS 1/2 medium. Neither the scarification methods nor the culture media significantly influenced the length of the radicle among seedlings cultivated in the light; under dark conditions the radicles grew statistically longer in MS ½ medium.

Acknowledgement

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PLANT SCIENCE

Antibacterial and cytotoxic activities of *Acacia aroma* extracts

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Abstract

Acacia aroma, native plant from San Luis, Argentina, is used commonly like antiseptic and healing of wounds. The present study was conducted to investigate the *in vitro* antibacterial activity of extracts of *A. aroma* and its toxicity on Vero cell. The bacterial strains used were *Staphylococcus aureus* ATCC 43300, *S. aureus* ATCC 35556, *Listeria monocytogenes* CLIP 74910 and *L. monocytogenes* CLIP 74904. The minimal inhibitory concentration (MICs) and minimal bactericidal concentration (CBMs) was determined and tested at concentration ranges from 5000 to 78µg/mL. On the other hand, the cytotoxic activity on Vero cells was assayed by MTT method. The MIC values of EE and HAE against *S. aureus* was 156µg/mL and 625µg/mL respectively. MBC values were one or two fold higher than the corresponding MIC values in both extracts. *Listeria* strains studied gave MICs and MBCs values of 78µg/mL and 312µg/mLl respectively for EE, while HAE showed less antibacterial activity against this strain (MIC=1250µg/mL and MBC=5000µg/mL). The CC50 was 658µg/mL for EE and 1020µg/mL for HAE. The extracts of *A. aroma* tested *in vitro* showed inhibitory activity against *Staphylococcus* and *Listeria*. These results allow validating the external use of this plant.

Key words: *Acacia aroma,* Antibacterial activity, Cytotoxicity

Introduction

Infectious diseases represent an important cause of morbidity and mortality among the general population, particularly in developing countries. Bacterial species presents the genetic ability to acquire and transmit resistance against currently available antibacterials. These antimicrobial-resistant bacteria are the causes of numerous clinical problems worldwide and the development and increase of resistance among pathogens causing nosocomial and community-acquired infections and are associated with the widespread utilization of antibiotics (Harvey and Gilmour, 2001; Ruiz-Bolivar et al., 2008; Sakoulas and Moellering, 2008; Howden et al., 2010). Due to the constant emergence of microorganisms resistant to conventional antimicrobials, the undesirable side effects of certain antibiotics and the emergence of

previously uncommon infections, is very important to develop new antimicrobial drugs especially from natural products.

In developing countries, a large proportion of the population utilizes medicinal plants for the treatment of infectious diseases. In many places in Argentina is a rich tradition of using herbal medicine for the treatment of various infectious diseases, inflammations, injuries (Zampini et al., 2007; Mattana et al., 2010). Considering the vast potentiality of plants as sources for antimicrobial drugs several authors have investiged the antimicrobial activity of medicinal plants (Srinivasan et al., 2001; Kumarasamy et al., 2002; Masika and Afolayan, 2002; Hamill et al., 2003).

The genus *Acacia*, one of the important genera of the family *Fabaceae* includes aproximately 1350 species and is abundant in Australia, Africa, India and America. Number biological activities have been reported from various species of *Acacia* (Akhtar and Khan, 1985; Li et al., 2003; Solomón and Shittu, 2010; Ramli et al., 2011; Lakshmit et al., 2011). *Acacia aroma* Gill. Ex Hook et Arn, which common name is tusca, is a native plant of Argentina, widely distributed in central and northwest region (Burkat, 1952). This plant is used

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in Argentina folkloric medicine as wound healing, antiseptic and for the treatment of gastrointestinal disorders.

Phytochemistry studies on *Acacia aroma* indicated that the main components of the steam volatile flower oils were methyl salicylate and eugenol. Furthermore, fatty acid profiles of *A. aroma* seed were examined (Lamarque et al., 2000). Mattana et al. (2010) in TLC analysis revealed the presence of flavonoids and saponins. Antimicrobial activity of flavonoids and saponines has also been reported against methicillin-resistant *Staphylococcus aureus* (Alcaráz et al., 2000; Li et al., 2002; Soetan et al., 2006; Tanaka et al., 2007; Kannabiran et al., 2008).

There is little scientific information concerning the antimicrobial activity of *A. aroma*. In Argentina, have been reported only studies on the antimicrobial activity of this plant in Tucuman and San Luis (Arias et al., 2004; Mattana et al., 2010).

The purpose of the present study was to investigate antibacterial activity of hot aqueous and ethanolic extracts of leaves *A. aroma* against *Staphylococcus aureus* and *Listeria monocytogenes* and to determine the degree of toxicity of these extracts in Vero cells.

Materials and Methods
Plant material

Aerial parts of *A. aroma* were collected in January-March of 2010, in the Northwestern region of the province of San Luis, Argentina. Voucher specimens under the number 487, were deposited in the herbarium of the Botany Department, San Luis National University (UNSL). Leaves were used for the study.

Preparation of Acacia aroma extracts
Crude ethanol extracts (EE)

The *A. aroma* leaf powder was macerated in ethanol 95% (V/V) in a 1:3 proportion at room temperature, undergoing mechanical shaking for 4 h, followed by filtration. The extract obtained was concentrated in a rotavapor at 40°C and the vegetable residue was extracted twice again analogously, thereby obtaining the crude ethanol extract.

Hot aqueous extract (HAE)

The *A. aroma* dried and powdered leaves (30 g) were macerated in water (1,400 mL) at 70°C for 120 min. This process was repeated twice. The extract obtained was filtered and lyophilized.

To perform the assays *in vitro* the extracts were solubilised in distilled water and sterilized by filtration through a 0.2μ membrane filter (Microclar).

Microorganisms

The microorganisms used in this study were as followed: *Staphylococcus aureus* ATCC 43300, *Staphylococcus aureus* ATCC 35556, *Listeria monocytogenes* CLIP 74910 and *Listeria monocytogenes* CLIP 74904. All organisms were maintained in brain-heart infusion (BHI medium) containing 20% (v/v) glycerol at −20°C. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 Mc Farland.

Antibacterial activity
Determination of minimal inhibitory concentration (MIC)

The MICs of *A. aroma* hot aqueous extract (HAE) and ethanolic extract (EE) were determined by micro-well dilution in tripticase soy broth supplemented with 0.01% (W/V) of 2,3,5-triphenyltetrazolium chloride as visual indicator of bacterial growth (CLSI, 2011). The inicial inocula were diluted 10 times (10^7 CFU/mL). The extracts were dissolved in distilled water to the highest concentration to be tested (5000μg/mL), and then serial two-fold dilutions were made in concentration ranges from 5000 to 78 μg/mL. The 96-well plates were prepared by dispensing into each well 95 μl of nutrient broth and 5 μL of the inoculum. One hundred microlitre aliquot from the stock solutions of the extracts and their serial dilutions initially prepared was transferred into seven consecutive wells. The final volume in each well was 200 μL. The plates were incubated at 37°C for 24 h. Controls were included. MIC was defined as the lowest concentration of the extracts in the medium in which there was no visible growth. The assays were performed in duplicate and then replicate at least twice.

Determination of minimal bactericidal concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bacterial growth. MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37°C.

Cytotoxicity assay
Cell culture

Cytotoxic assays were performed in Vero cells (*Cercopithecus aethiops* green monkey kidney epithelial cell line; ATCC CCL-81) grown in Eagle's minimal essential medium (EMEM) (Gibco, USA),

supplemented with 10% (v/v) heat-inactivated fetal calf serum (Natocor, Argentina), glutamine (30 mg/mL) and gentamicin (50 mg/mL) (Sigma–Aldrich, Italy). Cell cultures were maintained at 37ºC in a 5% (v/v) CO_2 humidified atmosphere.

Determination of 50% cytotoxic concentration

For cytotoxicity assay, the cells were cultured in 96-well culture plates (Cellstar, Greiner Bio-One, Germany). After incubation for 24 h at 37ºC, cells were exposed to increasing concentrations of the extracts. Assays were carried out in triplicate. Monolayers incubated only with EMEM were used as cellular controls. The concentration of the extracts which reduced the viable cell number to 50% (CC_{50}) was determined by MTT method. This colorimetric assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. The CC_{50} was measured by the MTT method (Mosmann, 1983). Briefly, monolayers treated with extracts for 48 h at 37ºC were incubated with MTT solution for 4 h at 37ºC. Subsequently, the supernatant was removed and acid-isopropanol (0.04N HCl in isopropanol) was added. After gently shaking for 15 min, the absorbance was read on a multiwell spectrophotometer (Bio-Tek, ELx800) at 570 nm. The Optical Density (OD) was measured at 560nm using a microplate ELISA reader (Labsystems Multiskan MS, Finland). The Survival Fraction % (SF%) in the treated cultures was calculated from the OD, in relation to cultures controls, that represent the 100% viability:

$$SF\% = \frac{OD\ treated\ cells}{OD\ control} \times 100$$

Statistical analysis

The CC50 values were calculated from concentration-effect curves after nonlinear regression analysis based on Boltzmann sigmoideal curve by the software *GraphPadPrism 5.0*. The results represent the mean ± standard error of the mean values of three different experiments.

Results and discussion
Antibacterial activity

The antibacterial activity of aqueous and ethanolic extracts of leaves of *A. aroma* was assayed against *S. aureus* and *L. monocytogenes* by the broth microdilution method. The ethanolic extract of *A. aroma* was the most active against the microorganisms studied. The Table 1 shows MICs and MBCs values against *S. aureus* and *L. monocytogenes*. This extract inhibited the growth of both microorganism at concentration of 156 μg/mL and 78 μg/mL respectively. The MBC values were one or two fold higher than the corresponding MIC values (625 μg/mL and 312 μg/mL respectively). In order to elucidate whether the observed antibacterial effects were bactericide or bacteriostatic, MBC/MIC ratios were calculated. Extracts with ratios greater than 1 were considered as bacteriostatic, while the other extracts were bactericide. These data allow concluding that all MIC values were bacteriostatic. Higher concentrations of ethanol extract were required to have for bactericidal effect. The hot aqueous extract (HAE) of *A. aroma*, showed a lower degree of antibacterial activity as compared with ethanolic extract. The HAE showed good biological activity against *S. aureus* (MIC=625μg/mL) however, against both strains of *Listeria* the activity was lower (MIC=1250μg/mL) (Table 1).

Some authors established that results would be considered significant if MIC or CBM ≤ 200 μg/mL (Suffredini et al., 2006). Our results with EE are in accordance with those studies. Ethanolic extract exhibited a higher degree of antibacterial activity, compared to aqueous extract. This observation confirmed the evidence from a previous study which reported that alcohol is a better solvent for extraction of antimicrobial substances from medicinal plants than water (Rojas et al., 2006).

Table 1. Minimal inhibitory concentration (MIC) and Minimal bactericidal concentration (MBC) obtained for ethanolic (EE) and hot aqueous extracts (HAE) from *Acacia aroma*.

Microorganisms	MIC (µg/ml)		MBC (µg/ml)	
	EE	HAE	EE	HAE
S. aureus ATCC 43300	625	156	625	1250
S. aureus ATCC 35556	625	156	625	1250
L. monocytogenes CLIP 74910	78	1250	312	5000
L. monocytogenes CLIP 74904	78	1250	312	5000

The biological activities of any medicinal plant are direct reflections of the effect and nature of the phytochemicals it contains. Previous phytochemical determination in ethanol and ethylacetate extracts from *A. aroma* done in our laboratory showed flavonoids and sapogenines compounds, and the bioautography assay demonstrated well-defined inhibition zones against *S. aureus* in correspondence with those flavonoids and sapogenines bands (Mattana et al., 2010). These phytochemicals are known to have various pharmacological activities including antimicrobial activity. Thus the presence of these compounds supports the traditional use of *A. aroma* in the treatment of wounds and skin infections.

Cytotoxic activity

The cytotoxic activity was carried out by using MTT assay. The results are graphically represented in Figures 1 and 2. Figure 1 shows the percentage of viability of Vero Cells, incubed 48 h in presence of ethanolic extract of *A. aroma* (EE) employed different concentrations. In this study, it was found that the 50% cytotoxic concentration (CC50) value was 658μg/mL for EE. This extract was not cytotoxic to Vero cells at bacteriostatics and bactericidal concentrations for all microorganisms tested.

EE *Acacia aroma*
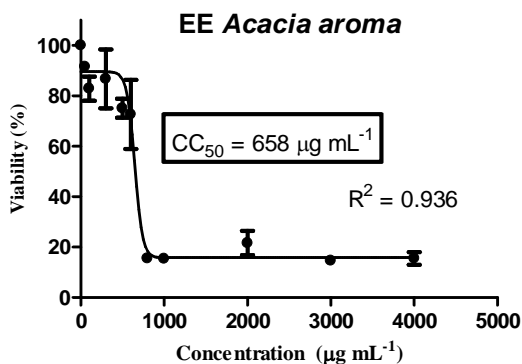
$CC_{50} = 658 \ \mu g \ mL^{-1}$

$R^2 = 0.936$

Figure 1. Percentage of viability of cultured Vero cells, incubated 48 h in the presence of ethanolic extract of *Acacia aroma* (EE) employed at different concentrations determined by MTT assay.
Each point represents the mean of four independent trials; CC50 was 658 μg/mL.

The CC50 of hot aqueous extract of *A. aroma* was 1020 μg/mL (Figure 2). This concentration showed not inhibitory activity against *L. monocytogenes,* however inhibited the growth of *S. aureus.*
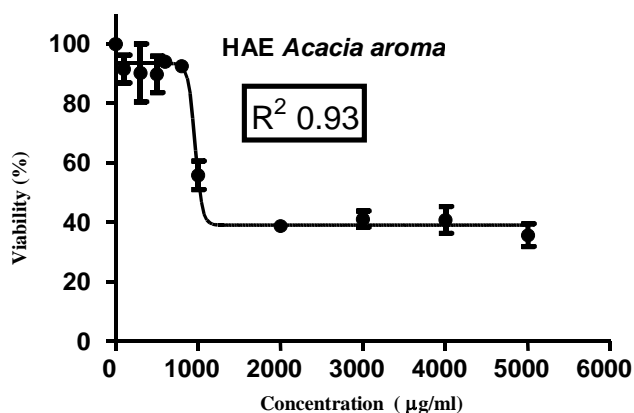

HAE *Acacia aroma*

$R^2 \ 0.93$

Figure 2. Percentage of viability of cultured Vero cells, incubated 48 h in the presence of hot aqueous extract (HAE) of *Acacia aroma* employed at different concentrations determined by MTT assay.
Each point represents the mean of four independent trials; CC50 was 1020 μg/mL.

Concentrations below 1000 μg/mL are considered non-toxic (Santos et al., 2003). Our results suggest that extracts tested exhibited selective toxicity, that means it present bactericidal or bacteriostatic effect without affect the eukaryotic cells. From the above results, it is concluded that these studies validate the external use of *A. aroma* extracts as complementary or alternative drugs to combat pathogenic microorganisms. Further study of separation and identification of bioactive principles and their evaluation *in vivo* models could clarify the properties of *A. aroma.*

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PLANT SCIENCE

Effect of wood ash, poultry manure and NPK fertilizer on soil and leaf nutrient composition, growth and yield of okra (*Abelmoschus esculentus*)

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Abstract

The data on poultry manure (PM), wood ash (WA) and NPK fertilizer are needed to identify strategies for sustainable management of a degraded Alfisol for improving agronomic productivity. Hence field experiments were conducted at Owo in the forest-savanna transition zone of southwest Nigeria to study the effects of organic amendments and NPK fertilizer on the soil chemical properties, leaf nutrient concentrations, growth and pod yield of okra. Seven treatments considered in 2006, 2007 and 2008 were control (no manure/fertilizer), 5.0 t ha^{-1} PM, 10.0 t ha^{-1} PM, 5.0 t hav WA, 10.0 t ha^{-1} WA, 5.0 t ha^{-1} PM + 5. 0 t ha^{-1} WA and NPK 15-15-15 (200 kg ha^{-1}) fertilizer. The treatments were laid out in a randomized complete block design with three replications. Organic amendments (PM and WA) improved soil organic C, N, P, K, Ca and Mg and leaf N, P, K, Ca and Mg concentrations, growth and yield of okra compared with the control. NPK 15-15-15 fertilizer increased soil N, P and K, but did not increase soil organic C, Ca and Mg and leaf Ca and Mg of okra. Combined application of 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA mostly improved leaf and soil N, P, K, Ca and Mg and soil organic C, growth and okra yield compared with other treatments. The superior performance of 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA was adduced to increased availability of nutrients following the inclusion of PM which aided faster decomposition and release of nutrients. Relative to the control, using the mean of the 3 years, 5.0 t ha^{-1} WA, 5.0 t ha^{-1} PM, NPK fertilizer, 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA, 10.0 t ha^{-1} WA and 10.0 t ha^{-1} PM increases pod yield by 23, 64, 68, 255, 41 and 123%, respectively. Combined use of PM and WA is recommended for ameliorating degraded Alfisol of southwest Nigeria and also reduced the quantities of PM and WA required for soil fertility maintenance.

Key words: Poultry manure, Wood ash, NPK fertilizer, Okra (*Abelmoschus esculentus*)

Introduction

Land degradation is regarded as an important global issue for the 21st century as it affects the environment, agronomic productivity and food security (Eswaran et al., 2001). The processes of soil degradation include the loss of top soil by water or wind, chemical deterioration of the natural resource which include among others, the reduction of soil biodiversity (Lal, 2001). According to Kirchhof and Salako (2000) and Salako et al. (2001), the impact of such soil degradation is difficult to reverse, especially if severe. Hence, early intervention in the rehabilitation of degraded soils is very important in achieving quick positive results and reversing the trend of degradation (Agbede and Ojeniyi, 2009). Furthermore, the

avoidance of soil loss by improved management of the natural resource is important to combat low agricultural production, food insecurity and the rapid increase in level of poverty (Ehui and Pender, 2005).

The majority of Alfisols available for crop production in the tropics have low organic matter and nutrient status. Consequently, intensive crop production requires high rate of organic and inorganic fertilizers. In the last fifteen years in Nigeria, chemical fertilizers have become very scarce and prices are high for average farmers. Hence, there is the need to search for organic amendments which could be utilized as fertilizer. Such amendments should be readily available, environmentally friendly and cheap. Integrating such amendments with one another might be the key to attaining the good yield. The effects of integrated nutrient supply as opposed to sole application of soil amendments in its effects on soil chemical properties, leaf nutrient concentration, growth and yield of okra has not been investigated,

although studies were conducted on the responses of okra to sole application of wood ash and poultry manure; For instance, Ojeniyi (2007) found increased in pod yield of okra with application of wood ash up to 8 t ha^{-1}. Likewise, Alasiri and Ogunkeyede (1999) reported that application of poultry manure at the rate of 10 t ha^{-1} gave the optimum seed yield of okra in southwest Nigeria.

Analysis of different organic amendments (Ojeniyi, 2007; Agbede and Ojeniyi, 2009) showed that poultry manure had high N and low K and C:N ratio values, and wood ash had high K and C:N ratio. It is expected that integrating the two organic amendments would have better effect on soil chemical properties, growth and yield of okra than the sole application of either of them. More so, the quantities of the organic amendments available may not be sufficient for large-scale okra production. Based on foregoing, Adediran et al. (2003) suggested the need for the combination of both the poultry manure and wood ash. Therefore this work investigated the sole and combined applications of poultry manure and wood ash compared with NPK-15-15-15 fertilizer on soil nutrient concentrations, leaf nutrient status, growth and yield of okra at Owo, southwest Nigeria.

Materials and Methods
Site description

A 3 year field experiment was carried out at Owo (latitude 7°12'N, longitude 5°35'E) within the forest-savanna transition zone of southwest Nigeria. The soil at Owo belongs to the broad group of Alfisol classified as Oxic Tropuldalf (USDA, 1999) or Luvisol (FAO, 1998) of the basement complex and locally classified as Okemesi Series (Smyth and Montgomery, 1962). The upper horizon (0-15 cm) of the experimental site is of sandy loam textural class. The values of the physical and chemical properties of the surface soil (0-15 cm) prior to experimentation are shown Table 1. This forest-savanna zone has a bimodal pattern of rainfall with first season commencing from April to July and a dry spell in August followed by second season in September to November. The annual rainfall totals were 1241, 1335 and 1442 mm for 2006, 2007 and 2008, respectively. The site of the experiment was fairly degraded and was dominated by Elephant grass (*Pennisetum purpureum*) and Guinea grass (*Panicum maximum*), but was left to fallow for 2 years before the start of the experiment. The same treatment was allotted to each plot for the 3 years of study.

Field experiment and treatments

The trial consists of sole and combined applications of poultry manure (PM) and wood ash (WA), and application of NPK-15-15-15 fertilizer at the rate of 200 kg ha^{-1}. The treatments compared for the 3 years were (a) control, i.e. no application of PM, WA or NPK-15-15-15 fertilizer, (b) 5.0 t ha^{-1} PM, (c) 10.0 t ha^{-1} PM, (d) 5.0 t ha^{-1} WA, (e) 10.0 t ha^{-1} WA, (f) 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA and (g) NPK-15-15-15 fertilizer at the rate of 200 kg ha^{-1}. The seven treatments were laid out in a randomized complete block design with three replications. After the initial clearing of the site and packing away of debris (no burning was done), manual ridges were constructed with a hoe.

Table 1. Mean ± standard deviation of soil physical and chemical properties (0-15 cm depth) at the experimental site in 2006 before the start of the experiment.

Property	Value
Sand (g kg^{-1})	690 ± 7.4
Silt (g kg^{-1})	130 ± 5.2
Clay (g kg^{-1})	180 ± 3.8
Soil texture	Sandy loam
pH (H$_2$0)	6.2 ± 0.2
Bulk density (Mg m^{-3})	1.54 ± 0.04
Total porosity (%, v/v)	41.9 ± 0.3
Organic C (g 100g^{-1})	1.30 ± 0.06
Total N (g 100g^{-1})	0.13 ± 0.02
Available P (mg kg^{-1})	6.2 ± 0.08
Exchangeable K (cmol kg^{-1})	0.07 ± 0.08
Exchangeable Ca (cmol kg^{-1})	1.7 ± 0.04
Exchangeable Mg (cmol kg^{-1})	0.12 ± 0.01

Crop establishment

The experimental plot size each year was 6 m x 5 m. Three okra seeds were planted at 60 cm x 60 cm. Okra variety NHAE-47-4 obtained from National Horticultural Research Institute, Ibadan, Nigeria was planted in May each year. Thinning to one plant per stand was done two weeks after sowing (WAS). Sole and combined application of PM and WA and NPK fertilizer was done immediately after thinning by ring method, thoroughly worked into the soil with a hoe. Manual weeding with cutlass was done twice in each experiment. Insect pests were controlled by spraying cypermethrin weekly at the rate of 30 ml per 10 litres of water from 2 WAS till 4 WAS (Adeyemi et al., 2005). The same treatment method was used on each plot for the 3 years of the study.

Soil sampling and analysis

Before the start of the experiment (after land clearing), 10 core soil samples, randomly collected from 0-15 cm depth were thoroughly mixed inside a plastic bucket to form a composite which was later

analysed for physical and chemical properties. At harvest in 2007 and 2008, another set of composite samples were collected per plot and similarly analysed for routine chemical analysis as described by Carter (1993). The soil samples were air-dried and sieved using a 2-mm sieve before making the determinations. Soil organic C was determined by the procedure of Walkley and Black using the dichromate wet oxidation method, total N was determined by micro-Kjeldahl digestion method, available P was determined by Bray- 1 extraction followed by molybdenum blue colorimetry. Exchangeable K, Ca and Mg were extracted using 1.0 N ammonium acetate. Thereafter, K was determined using flame photometer and Ca and Mg were determined using the EDTA titration method. Soil pH was determined in soil-water (1:2) medium using the digital electronic pH meter. Particle-size analysis was done using hydrometer method. Ten core samples (4 cm diameter, 10 cm high) were collected randomly before the start of the experiment for the determination of bulk density using the core method (Campbell and Henshall, 1991).

Okra leaf analysis

In 2007 and 2008, at 7 weeks after treatment application, leaf samples were collected randomly from each plot, oven-dried for 24 h at 80°C and ground in a Wiley-mill. The samples were analysed for leaf N, P, K, Ca and Mg as described by Tel and Hargarty (1984). Leaf N was determined by the micro-Kjeldahl digestion method. Samples were dry ashed at 500°C for 6 h in a furnace and extracted using nitric-perchloric-sulphuric acid mixture for determination of P, K, Ca and Mg (AOAC, 1990). Phosphorus was determined colorimeterically by the vanadomolybdate method, K was determined using a flame photometer and Ca and Mg were determined by the EDTA titration method.

Preparation and chemical analysis of organic amendments

The wood ash (WA) used for the study was collected from a bakery at Owo and was sieved with 2-mm sieve before application. The poultry manure (PM) used was stacked for 1 week under a shed to allow for mineralization. Small samples from the PM and WA used for the study were taken for laboratory analysis to determine their nutrient compositions. The samples were analysed for organic C, N, P, K, Ca and Mg as described by Okalebo et al. (1993).

Okra growth and yield

Twenty plants were randomly selected per plot for the determination of plant height, leaf area and pod yield. Plant height (by rule measure) and leaf area (by graphical method) were determined at 7 weeks after treatment application. Edible pods were harvested at 4 days interval and weighed. Pod weight was evaluated based on the cumulative number of pods at 8 harvests.

Statistical analysis

The data collected were subjected to analysis of variance and treatment mean were compared using Duncan's multiple range test (DMRT) at $p = 0.05$ probability level (Steel et al., 1997).

Results

The data presented in Table 1 are the results of the physical and chemical analysis of the experimental site before the start of the experiment in 2006. The soil was sandy loam and slightly acidic. The soil organic C and all the essential elements, i.e. N, P, K, Ca and Mg were very low according to the critical levels of 3.0% OM, 0.2% N, 10.0 mg kg^{-1} available P, 0.16 – 0.20 cmol kg^{-1} exchangeable K, 2.0 cmol kg^{-1} exchangeable Ca, 0.40 cmol kg^{-1} exchangeable Mg recommended for crop production in ecological zones of Nigeria (Akinrinde and Obigbesan, 2000). It will therefore unable to sustain crop yield without the addition of external input.

Table 2. Chemical composition of the organic amendments used for the experiment.

Parameters	Wood ash	Poultry manure
pH	11.1	6.8
Organic C (%)	18.0	29.5
Nitrogen (%)	1.72	4.31
C : N	10.5	6.8
Phosphorus (%)	0.86	0.83
Potassium (%)	3.6	2.2
Calcium (%)	8.6	1.4
Magnesium (%)	1.9	0.58

Table 2 shows the chemical composition of organic amendments used for the experiment. The pH of WA was very strongly alkaline and that of PM was slightly acidic. Except for organic C, N and P, WA has higher values of nutrients compared to PM. Poultry manure (PM) have low C:N ratio compared with WA. The amendments have higher values of organic C, P, N and cations than the soil used for the experiment. Hence, it is expected that the soil amendments used would improve the fertility of the soil and okra yield.

Effects of organic amendments and NPK-15-15-15 fertilizer on soil chemical properties

The effects of organic amendments and NPK-15-15-15 fertilizer on soil chemical properties in 2007 and 2008 are shown in Table 3. In the 2 years considered and relative to the control, sole applications of WA and PM increases and decreases soil pH linearly with rates of WA and PM applied respectively. There were no significant differences between the pH values of control, 5.0 t ha^{-1} PM and NPK-15-15-15 fertilizer and between 5.0 t ha^{-1} WA and combined applications of PM and WA (5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA).

Relative to the control, sole and combined applications of PM and WA significantly increased (p = 0.05) soil organic carbon (SOC) with 10.0 t ha^{-1} PM producing the highest value. NPK-15-15-15 fertilizer did not increase SOC. Relative to the control and using the mean of the 2 years, 10.0 t ha^{-1} PM, 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA, 5.0 t ha^{-1} PM, 10.0 t ha^{-1} WA and 5.0 t ha^{-1} WA, increased SOC by 241, 192, 113, 102 and 47%, respectively. Sole application of PM significantly gave higher SOC than the sole WA.

Compared with the control, organic amendments (PM and WA) significantly increased (p = 0.05) soil N, P, K, Ca and Mg concentrations. Whereas, NPK fertilizer significantly increased (p = 0.05) soil N, P and K, but did not increase soil Ca and Mg. Combined application of 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA produced the highest values of P, K, Ca and Mg concentrations. Phosphorus values of NPK fertilizer and 10.0 t ha^{-1} PM were not significantly different. Likewise, K values of NPK fertilizer and 10.0 t ha^{-1} WA were not significantly different. Sole application of PM and WA increased the concentration of organic C, N, P, K, Ca and Mg compared with the control. The values of these nutrients increased with increases in amount of WA and PM. Comparing the same sole rate of PM and WA, poultry manure (PM) significantly increased soil organic C, N and P concentrations relative to wood ash (WA). Wood ash (WA) significantly increased K, Ca and Mg concentrations relative to poultry manure (PM).

Effects of organic amendments and NPK fertilizer on leaf nutrient concentrations of okra

Compared to control, organic amendments significantly increased (p = 0.05) leaf N, P, K, Ca and Mg concentrations. Whereas NPK fertilizer significantly increased (p = 0.05) leaf N, P and K, but did not increase leaf Ca and Mg (Table 4). However, there were no significant differences in the leaf N of NPK fertilizer and 10.0 t ha^{-1} WA, and between 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA and 10.0 t ha^{-1} PM. Combined use of PM + WA produced the highest values of leaf K, Ca and Mg concentrations compared with other treatments.

Effects of organic amendments and NPK fertilizer on growth and yield of okra

The effects of organic amendments and NPK fertilizer on growth and yield of okra is shown in Table 5. All the soil amendments significantly increased (p = 0.05) plant height, leaf area per plant and pod yield of okra relative to control. Combined use of 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA gave the highest values of growth and yield parameters. There were no significant differences in the plant height, leaf area and pod yield of 5.0 t ha^{-1} PM and NPK fertilizer. Sole application of PM significantly recorded better plant height, leaf area and pod yield compared with sole WA. Likewise, the growth and yield parameters increases with amounts of PM and WA applied. Relative to the control, using the mean of the 3 years, 5.0 t ha^{-1} WA, 5.0 t ha^{-1} PM, NPK fertilizer, 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA, 10.0 t ha^{-1} WA and 10.0 t ha^{-1} PM increases pod yield by 23, 64, 68, 255, 41 and 123%, respectively. Compared with NPK fertilizer, 10.0 t ha^{-1} PM and 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA improved pod yield of okra by 32 and 111%, respectively.

Discussion

The increase in soil organic C, N, P, K, Ca and Mg concentrations due to the application of organic amendments and NPK fertilizer is consistent with the analysis recorded for the PM and WA in the present work and the use of PM, WA and NPK fertilizer for improving soil fertility in crop production (Patterson et al., 2004; Nottidge et al., 2005; Adenawoola and Adejoro, 2005). The decrease in pH with amount of PM could be due to the humic acid developed and CO_2 evolved in the process of decomposition of poultry manure. Chang et al. (1991) also found that pH in the surface 60 cm of non-irrigated and 90 cm of irrigated soil decreased with increased manure rates. Likewise, the findings that WA led to increase in soil pH is consistent with the analysis recorded for the pH of the wood ash used in the present study (pH of 11.1). Hence wood ash being a calcium-containing mineral raised the soil pH. The increase in soil pH observed in this treatment could be attributed to increased availability of calcium ions released into the soil solution during the microbial decarboxylation of wood ash which is known to buffer change in soil pH. This confirmed that wood ash improved the base status of the soil to which it is applied.

Table 3. Effects of organic amendments (PM and WA) and NPK fertilizer on soil chemical properties (0-15 cm depth) in 2007 and 2008.

Treatment	pH (H$_2$0)			Organic C(g 100g^{-1})			N (g 100g^{-1})			P (mg kg^{-1})			K (cmol kg^{-1})			Ca (cmol kg^{-1})			Mg (cmol kg^{-1})		
	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean
Control	6.1d	6.1d	6.1d	1.00f	0.92f	0.96f	0.09e	0.08e	0.09e	4.3f	4.2f	4.3f	0.06g	0.05g	0.06g	1.21f	1.00f	1.11f	0.10f	0.10f	0.10f
5.0 t ha^{-1} PM	5.8d	5.8de	5.8de	2.00c	2.08c	2.04c	0.38c	0.38c	0.38c	5.9d	5.9d	5.9d	0.26f	0.26f	0.26f	2.40e	2.60e	2.50e	0.14e	0.15e	0.15e
10.0 t ha^{-1} PM	5.3f	5.3f	5.3f	3.20a	3.33a	3.27a	0.71a	0.72a	0.72a	11.1b	11.1b	11.1b	0.48d	0.49d	0.49d	4.40c	4.45c	4.43c	0.26c	0.27c	0.27c
5.0 t ha^{-1} WA	7.3b	7.3b	7.3b	1.40e	1.41e	1.41e	0.21d	0.23d	0.22d	5.4e	5.4e	5.4e	0.37e	0.38e	0.38e	2.95d	3.01d	2.98d	0.23d	0.24d	0.24d
10.0 t ha^{-1} WA	8.1a	8.2a	8.2a	1.90cd	1.98cd	1.94cd	0.48b	0.46b	0.47b	8.6c	8.5c	8.6c	0.73b	0.75b	0.74b	5.61b	5.83b	5.72b	0.41b	0.41b	0.41b
5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA	7.0bc	6.9bc	7.0bc	2.75b	2.85b	2.80b	0.68a	0.70a	0.69a	12.5a	12.5a	12.5a	0.85a	0.86a	0.86a	6.42a	6.60a	6.51a	0.47a	0.47a	0.47a
NPK 15-15-15 (200 kg ha^{-1})	6.0d	5.9de	6.0d	0.96f	0.90f	0.93f	0.47b	0.47b	0.47b	11.0b	11.6b	11.3b	0.67bc	0.69bc	0.68bc	1.26f	1.03f	1.15f	0.09g	0.09g	0.09g

Means followed by similar letters are not significant different according to Duncan's multiple range test (DMRT); PM = Poultry manure; WA = Wood ash

Table 4. Effects of organic amendments (PM and WA) and NPK fertilizer on leaf nutrient concentrations of okra in 2007 and 2008.

Treatment	N (g 100g^{-1})			P (g 100g^{-1})			K (g 100g^{-1})			Ca (g 100g^{-1})			Mg (g 100g^{-1})		
	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean	2007	2008	Mean
Control	1.40e	1.39e	1.40e	0.09f	0.08e	0.09f	1.00f	1.09f	1.00f	0.30f	0.30f	0.30f	0.09f	0.08f	0.09f
5.0 t ha^{-1} PM	2.55c	2.56c	2.56c	0.13d	0.12c	0.13d	1.15d	1.15d	1.15d	0.51e	0.51e	0.51e	0.11e	0.11e	0.11e
10.0 t ha^{-1} PM	4.90a	4.95a	4.93a	0.28a	0.29a	0.29a	1.98c	1.98c	1.98c	0.79c	0.81c	0.80c	0.15c	0.15c	0.15c
5.0 t ha^{-1} WA	1.66d	1.69d	1.68d	0.11e	0.10d	0.11e	1.30e	1.31e	1.31e	0.61d	0.61d	0.61d	0.13d	0.12d	0.13d
10.0 t ha^{-1} WA	3.60b	3.60b	3.60b	0.15c	0.16b	0.16c	2.50b	2.55b	2.53b	1.00b	1.00b	1.00b	0.21b	0.21b	0.21b
5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA	4.80a	4.95a	4.88a	0.29a	0.28a	0.29a	2.82a	2.96a	2.89a	1.28a	1.26a	1.27a	0.25a	0.25a	0.25a
NPK 15-15-15 (200 kg ha^{-1})	3.70b	3.60b	3.70b	0.26b	0.28a	0.27b	2.45b	2.47b	2.46b	0.30f	0.30f	0.30f	0.09f	0.08f	0.09f

Means followed by similar letters are not significant different according to Duncan's multiple range test (DMRT); PM = Poultry manure; WA = Wood ash.

Table 5. Effects of organic amendments (PM and WA) and NPK fertilizer on plant height, leaf area and pod weight of okra in 2006, 2007 and 2008.

Treatment	Plant height (m)				Leaf area per plant (m^2)				Pod weight (t ha^{-1})			
	2006	2007	2008	Mean	2006	2007	2008	Mean	2006	2007	2008	Mean
Control	0.32f	0.34f	0.33f	0.33f	0.70f	0.74f	0.72f	0.72f	2.4f	2.2f	2.1f	2.2f
5.0 t ha^{-1} PM	0.48c	0.49c	0.50c	0.49c	1.11c	1.18c	1.10c	1.13c	3.6c	3.6c	3.5c	3.6c
10.0 t ha^{-1} PM	0.52b	0.58b	0.59b	0.56b	1.25b	1.31b	1.28b	1.28b	4.8b	4.9b	4.9b	4.9b
5.0 t ha^{-1} WA	0.39e	0.41e	0.40e	0.40e	0.90e	0.92e	0.91e	0.91e	2.7e	2.8e	2.6e	2.7e
10.0 t ha^{-1} WA	0.44d	0.46d	0.47d	0.46d	0.99d	1.04d	1.00d	1.01d	3.0d	3.1d	3.2d	3.1d
5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA	0.59a	0.64a	0.65a	0.63a	1.60a	1.66a	1.52a	1.59a	7.7a	7.8a	7.9a	7.8a
NPK 15-15-15 (200 kg ha^{-1})	0.49c	0.51c	0.50c	0.50c	1.11c	1.19c	1.10c	1.13c	3.7c	3.7c	3.6c	3.7c

Means followed by similar letters are not significantly different according to Duncan's multiple range test (DMRT); PM = Poultry manure; WA = Wood ash.

Park et al. (2004) also found that WA significantly increased soil pH in the 0-10 cm soil layer from 6.1 in the control to 6.9 and 7.1 in the 10 and 20 Mg ha^{-1} plots, respectively.

The findings that combined application of PM and WA (5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA) gave the highest values of P, K, Ca and Mg is attributable to release of nutrients from both the WA and PM. The integration of the PM with the WA should have enhanced faster decomposition and release of nutrients from the WA due to low C:N ratio of the PM. The lower soil nutrient concentrations of NPK fertilizer in spite of its high nutrient value could be adduced to leaching and erosion. The findings that sole PM significantly increased soil organic C, N and P and low K, Ca and Mg concentrations compared with WA is consistent with the analysis of nutrients for PM and WA.

The significant increase in leaf N, P, K, Ca and Mg concentrations of okra by the application of either sole or combined PM and WA is attributable to increased availability of nutrients in soil by the application of the soil amendments leading to increased uptake by okra plant. It was found that 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA produced significantly higher values of leaf K, Ca and Mg concentrations compared with other treatments. This could be attributed to increased microbial activities and mineralization of nutrients induced by poultry manure addition. NPK fertilizer increased leaf N, P and K, but did not increase leaf Ca and Mg, hence the fertilizer and control gave similar leaf Ca and Mg concentrations.

The findings that all amendments and NPK fertilizer increased plant height, leaf area and pod yield of okra is consistent with the low soil organic C, N, P, K, Ca and Mg status of the soil before the start of the experiment. This observation confirmed the importance of fertilizer or organic amendments in promoting okra performance in a degraded Alfisol

of the humid tropics. The findings that 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA produced better growth and pod yield of okra is adduced to the fact that the PM with low C:N ratio tended to decompose fast and release nutrients for crop uptake. It also improved microbiological activity and enhanced soil fertility status. Therefore its addition to WA would have reduced nutrient immobilization due to high C:N ratio and enhanced released of nutrient from the WA. The superior plant height, leaf area and pod yield of okra given by 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA compared to other treatments could also be attributed to the increased in N and K concentrations of the okra plant. Majanbu et al. (1986) had shown that N and K are the most important macronutrients that okra required for proper growth and pod production. Poultry manure is known to have high concentrations of N and P and low C:N ratio while WA is high in K with high C:N ratio, this attribute of PM would have enhanced faster decomposition of WA and quicker release of nutrients for okra plant uptake, hence better growth and yield of okra with 5.0 t ha^{-1} PM + 5.0 t ha^{-1} WA than either sole WA or PM application and NPK fertilizer.

Conclusion

Sole and combined applications of WA and PM and NPK fertilizer improved soil fertility status of a degraded Alfisol as indicated by increase in soil organic C, N, P, K, Ca and Mg and leaf N, P, K, Ca and Mg concentrations of okra plant. Combined applications of WA and PM mostly improved soil fertility and leaf K, Ca and Mg, growth and pod yield of okra. Plant (WA) and animal (PM) wastes could be combined and used as organic amendments and be well substituted for expensive and scarce NPK fertilizer in improving the fertility status of a degraded Alfisol in the forest-savanna transition zone of southwest Nigeria.

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PLANT SCIENCE

Zinc in plants - An overview

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Abstract

Owing to continuous development of knowledge at a biophysics, biochemistry, molecular biology and genetics levels, essential progresses to understand the mechanisms underlying the threshold of Zn toxicity has been achieved. Following this background, Zn uptake and translocation and its functions in plants are described, being its toxic effect on growth, photosynthetic activity and oxidative stress discussed. The extent of plant injury by elevated Zn concentrations is also assessed, considering its specific and strong dependence on the environmental conditions and availability of other heavy metals.

Key words: Photosynthesis, Pollution, Stress, Threshold of toxicity, Zinc

Abbreviations: ATP = Adenosine-5'-triphosphate; CA = Carbonic anhydrase; CDF = Cation diffusion facilitator; Chl = Chlorophyll; DNA = Deoxyribonucleic acid; EPR = Electron paramagnetic resonance; Fm = Maximal chlorophyll fluorescence; F_o = Initial chlorophyll fluorescence; F_v = Variable chlorophyll fluorescence; F_v / F_m = Maximal photochemical efficiency; $\Delta F/F_m'$ = Quantum yield of electro flow throughout photosystem II; IAA = Indole-3-acetic acid; LHC = Light harvesting complex; NADPH = Nicotinamide adenine dinucleotide phosphate; NSCCs = Non selective cation channels; PEP = Phosphoenol pyruvate; PS = Photosystem; Q = Quinone; q_N = Non photochemical fluorescence quenching; qP = Photochemical fluorescence quenching; RC = Reaction centre; RNA = Ribonucleic acid; ROS = Reactive oxygen species; Rubisco = Ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP = Ribulose-1,5-bisphosphate.

Introduction

The amounts of Zn in unpolluted soils typically are lower than 125 ppm (Ebbs and Kochian, 1997; Di Baccio et al., 2003; Hussain et al., 2010) and in plants growing in these soils this metal concentration varies between 0.02-0.04 mg g^{-1} dry weight (Bowen, 1979). Moreover, the outrun of the Zn threshold of toxicity is mostly determined by environmental pollution following industrial and agricultural activities, such as smelter and incinerator emissions, dispersal from mine wastes, excessive applications of Zn-containing fertilizers or pesticides and use of Zn-contaminated sewage sludges, manures or industrial wastes as fertilizers

(Pedler et al., 2004; Giuffré et al., 2012).

The sources of Zn contamination in soils are often associated with Pb, Cu, and Cd (Pedler et al., 2004), yet its uptake and translocation rates in plants are higher than Cd (Chakravarty and Srivastava, 1997; Ali et al., 2000). Zn also becomes toxic at higher concentrations than the latter (Van Assche and Clijsters, 1986a,b). Moreover, the bioavailability of Zn in soil solution increases at low pH, while organic ligands and hardness cations such as Ca^{2+} decrease Zn availability (Pedler et al., 2004).

Zn is an essential micronutrient involved in a wide variety of physiological processes (Van Assche and Clijsters, 1986ab; Baker and Brooks, 1989; Marschner, 1995; Ali et al., 1999, 2000; Cakmak and Engels, 1999; Cakmak, 2000; Reeves and Baker, 2000; Doncheva et al., 2001; Stoyanova and Doncheva, 2002; Di Baccio et al., 2005; Broadley et al., 2007), yet at concentrations above 0.2 mg g^{-1} dry matter the potential phytotoxicity at leaf tissue develops (Ali et al., 2000; Bonnet et al., 2000). With phytotoxicity rising up reduced yields and stunted growth overcame (Marschner, 1995;

Broadley et al., 2007), chiefly due to the photosynthetic performance falls, being affected the photochemical reactions (Van Assche and Clijsters, 1986a), carbonic anhydrase activity (López-Millán et al., 2005), biosynthesis of Chls (Van Assche and Clijsters, 1986b) and cell membrane integrity (Wissemeier and Horst, 1987).

Considering that the extent of Zn phytotoxicity varies in a wide range, but mostly depends on plant species, age, environmental conditions and combinations with other heavy metals, in this review available data about physiological aspects of Zn in plants are summarized and integrated in a general metabolic perspective.

Zn Uptake and translocation

Zn uptake, although varying among plant species, is determined by the composition and concentration of the growth media. Zn uptake arises as a divalent cation or as complexes with organic ligands, tends to display a linear pattern with its concentration in the nutrient solution or in the soils (Thoresby and Thornton, 1979; Kabata-Pendias and Pendias, 2001), and via xylem the roots load the shoots tissues (Broadley et al., 2007).

Zn translocation to roots xylem occurs via symplast and apoplast (Brennan, 2005; Broadley et al., 2007), but high levels of Zn have also been detected in the phloem, denoting that this metal is translocated through both xylem and phloem tissues (Pearson et al., 1995; Haslett et al., 2001).

Conflicting reports state that Zn uptake is an active or passive process (Brennan, 2005). A decrease of Zn absorption in barley roots, under anaerobic conditions, by lowering temperatures and due to metabolic uncouplers, suggests that this metal uptake by plant roots follows an active process (Schmidt et al., 1965). Bowen (1969) also demonstrated that Zn absorption in the leaves of sugarcane is strongly depressed due to the inhibition of oxidative phosphorylation, whereas Bowen et al. (1974) implied that low temperatures inhibit the absorption of Zn in the roots of *Pinus radiate*. Moreover, as Zn^{2+} uptake does not respond to metabolic inhibitors, it has also been concluded that this process is not metabolically-dependent (Kochian, 1993). Independently of the type of uptake kinetics of Zn, as a fraction of this metal occurs bound to light organic compounds in xylem fluids, its high mobility in the plants is recognized (Tiffin, 1977; Kabata-Pendias and Pendias, 2001). Relatively to the highly mobile elements such as K or P and the immobile element Ca, Zn has an intermediate mobility (Marschner, 1995; Kabata-Pendias and Pendias, 2001).

Apart the kinetics of Zn^{2+} uptake, the mechanisms controlling its transport across the root and other cell membranes seems to be metabolically controlled. This process is driven through an electrochemical gradient via ion channels, carrier proteins or against the electrochemical gradient via electrogenic pumps (Weiss et al., 2004). These authors, following Hille (2001), reported that Zn^{2+} membrane transport is dominated by ion channels and electrogenic pumps, rather than by carrier-mediated transport. Under this upbringing, these ion channels, which are pore-forming proteins, can establish and control a voltage gradient across the plasma membrane of cells, allowing ions flow down their electrochemical gradient. Some channels also might let the passage of ions based solely on their positive or negative charge, whereas groupings of ion channels regulate the passage through the pore and can open or close by chemical or electrical signals and temperature. Additionally, non selective cation channels (NSCCs) have the capacity to catalyse passive fluxes of cations, namely Zn^{2+}, through plant membranes (Demidchik et al., 2002). NSCCs, which are a diverse group of ion channels in the plasma membrane, tonoplast, and other endomembranes, are gated by diverse mechanisms, that include voltage, cyclic nucleotides, glutamate, reactive oxygen species, and stretch. These structures are claimed to lighten the fluxes of a wide range of monovalent cations and several are also permeable to divalent cations including Zn^{2+} (Demidchik and Maathuis, 2007).

To assess the mechanisms of Zn^{2+} uptake and translocation in plant species, in the last 10-15 years rapid progress has been achieved upon consideration of the molecular mechanisms of metal transport across cell membranes. Through the application of genetic and molecular techniques, a wide range of gene families involved in heavy metal transport in plants have been identified (Hall and Williams, 2003). The first plant Zn transporter gene sequences were identified in *Arabidopsis thaliana*. These genes, from the family ZIP1-4 (zinc regulated ZRT/IRT-like transporter proteins), have been shown to be uninfluenced or downregulated by Zn fertilization (Grotz et al., 1998; Burleigh et al., 2003). A plant transporter gene from CDF family was firstly characterized in *Arabidopsis* and designated ZAT (van der Zaal et al., 1999). It was claimed that ZAT is involved in the vesicular/vacuolar sequestration of Zn and implicated in Zn homeostasis and tolerance (Hall and Williams, 2003). Afterward, ZIP-like Zn transporter genes from a second plant species,

Thlaspi caerulescens have been characterized (Pence et al., 2000). A gene encoding a plasma membrane localized Zn transporter named MtZIP2 was cloned from Medicago truncatula (Burleigh et al., 2003). Unlike any of the other plant Zn transporters, MtZIP2 has the unique feature of being up-regulated in roots by Zn fertilization. Hussain et al. (2004) provided evidence that HMA2 and HMA4 *Arabidopsis* transporters from P-type ATPases subfamily also play an important role in Zn transport and homeostasis in plants.

Biochemical functions of Zn

The metabolic functions of Zn are based on its strong tendency to form tetrahedral complexes with N- , O- and S-donor ligands (Marschner, 1995; Alloway, 2004). The predominant forms of Zn are low molecular weight complexes, storage metalloproteins, free ions, and insoluble structures associated with the cell walls. At an intracellular level Zn is inactivated throughout complexes with organic ligands or by complexation with phosphorus (Brown et al., 1993; Alloway, 2004).

Within plants Zn seems to affect the capacity for water uptake and transport (Barceló and Poschenrieder, 1990; Kasim, 2007; Disante et al., 2010) and to reduce the adverse effects of short periods of heat stress (Peck and McDonald, 2010) or of salt stress (Tavallali et al., 2010). Since Zn is required for the synthesis of tryptophan (Brown et al., 1993; Alloway, 2004), which is a precursor of IAA, this metal also has an active role in the production of auxin, an essential growth hormone (Skoog, 1940; Brennan, 2005). The involvement in signal transduction via mitogen-activated protein kinases was further reported (Lin et al., 2005; Hänsch and Mendel, 2009).

The integrity of cellular membranes also requires Zn to preserve the structural orientation of macromolecules and keep ion transport systems (Cakmak, 2000; Kabata-Pendias and Pendias, 2001; Alloway, 2004; Dang et al., 2010; Disante et al., 2010). The interaction of Zn with phospholipids and sulphydryl groups of membrane proteins further contributes for the maintenance of membranes.

As a prosthetic component of enzymes within cells, namely dehydrogenases, aldolases, isomerases, transphosphorylases, RNA and DNA polymerases (Kessler, 1961; Srivastrava and Gupta, 1996; López-Millán et al., 2005), Zn is implicated in protein synthesis and energy production (Hänsch and Mendel, 2009). It is also involved in nucleic acid synthesis, carbohydrate and lipid metabolisms

(Marschner, 1995) and it forms complexes with DNA and RNA, swaying their stability (Pahlsson, 1989; Coleman, 1992).

Following enzymes kinetics in the photosynthetic apparatus, proteolytic activities such as the repair processes of PSII, by turning over the photo-damaged D1 protein, are dependent of Zn (Bailey et al., 2002; Hänsch and Mendel, 2009). This nutrient also modulates the CA activity, and therefore the conversion of carbon dioxide to reactive bicarbonate species required for the fixation to carbohydrates. In addition, as high CA activity is a core requirement in the mesophyll chloroplasts, particularly in C_4 plants, Zn further has a central role to shift the equilibrium in favour of HCO_3^-, the substrate for PEP carboxylase (Graham and Reed, 1991; Marschner, 1995; Alloway, 2004). To maintain adequate HCO_3^- supply in the guard cells, Zn is also implicated in stomata opening, possibly as a constituent of CA (Sharma et al., 1995), whereas in parallel this metal determines the influx rate of K^+ in these cells (Brennan, 2005). The regulation of the biochemical reactions in the photosynthetic metabolism is also driven by Zn as this metal integrates the structure of Rubisco (Brown et al., 1993; Alloway, 2004).

In cellular membranes, namely in the thylakoid lamellae, some ligands, namely cysteine and histidine, can bind Zn with high affinity and more stability than to Fe (Berg and Shi, 1996), restricting the production of high toxic hydroxyl radicals through Haber-Weiss reactions (Cakmak, 2000; Alloway, 2004; Brennan, 2005; Disante et al., 2010). There are evidences that Zn is involved in oxidative stress-induced expression of genes encoding antioxidative defence enzymes, such as H_2O_2-scavenging ascorbate peroxidase and glutathione reductase (Alscher et al., 1997; Cakmak, 2000).

Toxic effects of excessive Zn on growth

Excess of Zn^{2+}, following a wide-ranging plant response to heavy metal stress (Ouariti et al., 1997; Prasad, 1999; Bonnet et al., 2000; Artetxe et al., 2002; Di Baccio et al., 2005), beyond prompting negative interferences with others nutrients uptake (Kaya et al., 2000) and enzyme activities (Ouariti et al., 1997; Khudsar et al., 2004), elicits wilting, necrosis of old leaves (Soares et al., 2001; Di Baccio et al., 2005), biomass decline and inhibition of cell elongation and division (Wallnofer and Engelhardt, 1984; Arduini et al., 1994; Cakmak, 2000; Khudsar et al., 2004). Moreover, the threshold of toxicity sharply varies among plant

species, time of exposure to Zn stress and composition of the nutrient growth medium. Growth inhibition develops in *E. maculata* and *E. urophylla* by five weeks after addition of 400-1600 mM $ZnSO_4$ (Soares et al., 2001), whereas *Pisum sativum* became inhibited after 1000 μM Zn application (Doncheva et al., 2001). In ryegrass, growth retardation occurs at 1-10 mM $ZnSO_4$, with a total growth inhibition developing at 50 mM $ZnSO_4$, (Bonnet et al., 2000). *Datura* species (*D. metel, D. innoxia, D. sanguinea,* and *D. tatula*) also become severely reduced after 20 days of Zn stress imposed by 2.5 and 5 mM $ZnSO_4$ (Vaillant et al., 2005). Cells from *Synechocystis aquatilis f. aquatilis* (*Cyanophyceae*) growing at high Zn levels (2.20-3.30 mg L^{-1}) show a decreased specific growth rate and a final yield of about 50-60%, relatively to cells grown in the presence of 0.21 mg Zn L^{-1} (De Magalhaes et al., 2004). Significant reductions in foliage and total dry mass of *Populus deltoides* x *Populus nigra* (*Populus x euramericana*) develop after applications of 100 μM and 1000 μM Zn, respectively (Di Baccio et al., 2003). Zn concentrations of 100 – 400 μg g^{-1}(soil d.m.) cause significant decrease in root and shoot growth parameters at different developmental stages of *Artemisia annua* plants (Khudsar et al., 2004). Shoots and roots elongation is also retarded in green grass (Veer and Lata, 1989), *Phaseolus mungoo* (Chaoui et al., 1997), *Vigna radiata* and *Sorghum bicolor* (Balashouri, 1995) and *Bacopa monniera* (Ali et al., 1999). Growth inhibition, chlorosis and necrosis develop (Kopponen et al., 2001) in birch clones (for instance, *Betula pendula* and *Betula pubescens*) tested in soils supplemented with Zn (0.5-5 g kg^{-1} d.m. soil). High doses of Zn (65 and 130 mg L^{-1}) can also trigger growth depression, dark green leaves, decreased root number and length and sharp depression in the mitotic activity of roots from sugarcane (Jain et al., 2010).

Photosynthesis breaking by Zn excess

Photosynthesis is strongly affected in plants exposed to heavy metals excess (Baszyński and Tukendorf, 1984; Clijsters and Van Assche, 1985; Baszyński, 1986; Stiborova et al., 1986; Prasad, 1999). Following the heterogeneous threshold of toxicity within plant species, different amounts of Zn compulsory can restrict the stomatal conductance (Singh and Bhati, 2003; Khudsar et al., 2004; Di Baccio et al., 2005; Vaillant et al., 2005; Dhir et al., 2008; Sagardoy et al., 2010) and therefore CO_2 fixation (Hampp et al., 1976; Van Assche et al., 1980, 1988; Khudsar et al., 2004),

whereas mesophyll tissues are affected in parallel with the number and size of stomata cells (Vaillant et al., 2005; Sagardoy et al., 2010). The mechanisms of stomatal response to high Zn concentration seem to be related to changes in CA activity, because Zn appears to have a regulatory influence on stomatal opening, possibly as a constituent of CA (Sharma et al., 1995). In this context, Hu et al. (2010) further claimed that the CO_2-binding CA proteins that catalyse the reversible transformation $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, might function in CO_2 signaling.

At molecular level the inhibition of the photosynthetic apparatus is couple with critical changes in Chl structure and amounts. Inside the Chl structure, Mg^{2+} can be replaced with heavy metals, namely by Zn (von Wettstein et al., 1995), which therefore impairs the functioning of LHCII (Kowalewska et al., 1992; Küpper et al., 1996, 1998, 2002). Toxic concentrations of Zn^{2+} also decrease the levels of Chl *a* and *b* as well as the *a*/*b* ratio (Khudsar et al., 2004; Vaillant et al., 2005; Cherif et al., 2010; Ivanov et al., 2011), but although inhibiting PSII and/or PSI photochemical efficiency (Di Baccio et al., 2003), differentially affects the LHCII, where Chl *b* is located, rather than the Chl *a* RC (Bertrand and Poirier, 2005). A possible obstruction in the transport of Fe to the chloroplasts seems to determine the reduction of the Chl content in the leaves (Clijsters and Van Assche, 1985; Symeonidis and Karataglis, 1992; Bekiaroglou and Karataglis, 2002).

In the photochemical reactions in the chloroplast lamellae, the inhibition of the photosynthetic electron transport (Tripathy and Mohanthy, 1980) prevails at the Hill reactions (Hampp et al., 1976) and implicates the water evolving complex of PSII (Moustakas et al., 1994). In the cluster of the water splitting complex Mn seems to be replaced by Zn (Ralph and Burchett, 1998), which inhibits photolysis and oxygen emission (Monnet et al., 2001). An active Zn^{2+} inhibitory site on the donor side of the PSII also arises (Tripathy and Mohanthy, 1980; Van Assche and Clijsters, 1986a; Rashid et al., 1991), since that ion directly modifies the Q_B site (Mohanty et al., 1989), affects the reduction of Q_A without changing the re-oxidation properties (Ciscato et al., 1999) and disturbs the conformation of the PSII core complex (Rashid et al., 1991), which also affects the acceptor side of the photosystem. Szalontai et al. (1999) further explain the inhibition of the electron transport mediated by Zn reporting alterations in the membrane architecture, mostly due to a smaller extent to heat-induced

denaturation. Among plant species the Chl fluorescence kinetics further indicates these inhibitory targets on the photosynthetic electron transport. Indeed, *Lolium perenne* plants submitted to different levels of $ZnSO_4$, show a net decline in the capacities of F_v/F_m and in $\Delta F/F_m'$ (Bonnet et al., 2000). Similar results were found with *Phaseolus* species (Krupa, 1993; Maksymiec and Baszynski, 1996). Szalontai et al. (1999) observed strong decreases in F_v and F_m in pea chloroplasts after Zn^{2+} treatment and considered this an evidence for inhibition of PSII activity. Plekhanov and Chemeris (2003) observed a suppressed relative yield of F_v/F_m after treatment of *Chlorella pyrenoidosa* with elevated Zn^{2+} concentrations, which indicates a rapid inactivation of PSII. These authors further analysed the induction curve of delayed Chl fluorescence in *Chlorella* cells and suggested that the early toxic effects of elevated Zn^{2+} concentrations manifested itself not only in inhibited electron transport of PSII, but also of a reduced photosynthetic membrane energization. Mallick and Mohn (2003) revealed that Zn and other heavy metals inhibited substantially PS II photochemistry after 12 h treatment of the green microalga *Scenedesmus*, which was clearly evident from F_0 and F_m, F_v/F_m, q_N and q_P. According to these authors, the plastoquionone pool and the water-splitting apparatus were significantly altered under metal stress. Nevertheless, according to Subrahmanyam and Rathore (2000), the observed decrease of PSII activity can partly be due to a down-regulation caused by a reduced demand for ATP and NADPH in the Calvin cycle.

In the stroma of the chloroplasts, the kinetics of the biochemical reactions is driven by Rubisco activity, which is sensitive to toxic amounts of Zn. Although bivalent cations play a major role in the activation of Rubisco and in the equilibrium between CO_2 and O_2 binding (Lorimer, 1981), Co^{2+}, Ni^{2+} and Zn^{2+} displace Mg^{2+} in the structure of Rubisco, which determines the loss of its activity (Van Assche and Clijsters, 1986b; Schützendübel and Polle, 2002; Mateos-Naranjo et al., 2008). Nevertheless, Zn mostly inhibits the carboxylase activity of this enzyme without affecting its oxygenase function (Van Assche and Clijsters, 1986ab).

Induction of oxidative stress by excessive zinc

Heavy metals trigger oxidative stress, inhibiting the basic physiological functions of plants (Clijsters and Van Assche, 1985; Van Assche and Clijsters, 1990; Lidon and Henriques, 1993; Szalontai et al., 1999; Maksymiec et al., 2008). Although redox-active metals, namely Fe and Cu, catalyze the formation of hydroxyl radicals through the Haber–Weiss reactions and cause direct oxidative damage as well as antioxidative responses (Tripathy and Mohanthy, 1980; Lidon and Henriques, 1993; Schutzendubel and Polle, 2002), Zn is unable to perform one electron oxidoreduction reactions (López-Millán et al., 2005). Neverthless, excess Zn generates oxidative stress (Madhava Rao and Sresty, 2000; López-Millán et al., 2005), producing toxic oxygen species, such as $O_2^{\bullet-}$ and H_2O_2 (Kappus, 1985; Weckx and Clijsters, 1997) and concomitantly inducing disruption of metabolic processes such as the antioxidant defence system or the photosynthetic electron transport (Cakmak, 2000). In this context, oxidative stress mediated by Zn excess increases lipids peroxidation and membrane permeability, diminishing the sulphydryl content (Tripathi and Gaur, 2004). Within this oxidative stress Zn stimulates lipoxygenase activities in parallel with lipids peroxidation, which possibly is linked to the activation of the plasma membrane NADPH oxidase, leading to increased superoxide anion production (Weckx and Clijsters, 1997). The mechanism for zinc-promoted oxidative stress in the leaf apoplast, root growth zone and xylem, also seems to be associated to the generation of charge transfer complexes and quinhydrone, because of phenoxyl radical stabilisation by Zn^{2+} in the cell wall or by inhibition of photosynthetic electron transport (Morina et al., 2010). Moreover, depending on Zn contents, the antioxidant system, namely superoxide dismutase, ascorbate peroxidase and glutathione reductase and the contents of ascorbic acid and total glutathione is also activated (Madhava Rao and Sresty, 2000; Jain et al., 2010; Cui and Zhao, 2011; Kleckerova et al., 2011; Ivanov et al., 2012).

Conclusion

The extent of plant injury by elevated Zn concentrations is specific and strongly depends on the environmental conditions and on the availability of heavy metals. Further elucidation of the mechanisms of Zn translocation and homeostasis especially the role of transporter families can favour the implementation of genetic modification in several species, to improve the fortification of Zn in crops for human and animal nutrition or even to further develop phytoremediation. In this context, protective mechanisms, namely avoidance, chelation and sequestration inside the cells, or

efflux from the cytosol to the apoplast, could be further understood. Thus, in soils containing high concentrations of Zn, these hyperaccumulators species could be cultivated, which would be beneficial for reducing the unusable Zn-polluted fields by the method of phytoremediation.

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PLANT SCIENCE

Ecological assessment of biotoxicity of pesticides towards plant growth promoting activities of pea (*Pisum sativum*)-specific *Rhizobium* sp. strain MRP1

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Abstract

This study was planned to assess the impact of pesticides [herbicides (metribuzin and glyphosate), insecticides (imidacloprid and thiamethoxam) and fungicides (hexaconazole, metalaxyl and kitazin)] at the recommended and the higher dose rates on plant growth promoting (PGP) traits of Rhizobium sp. strain MRP1 isolated from pea-nodules. Strain MRP1 was unambiguously selected due to high pesticide-tolerance and substantial production of indole acetic acid, siderophores, exo-polysaccharides, HCN and ammonia. Pesticide-concentration dependent progressive-decline for PGP properties of Rhizobium sp. strain MRP1 was observed except exo-polysaccharides which regularly increased on increasing concentration of each pesticide beyond the recommended dose. For example, hexaconazole at three times the recommended dose decreased salicylic acid and 2, 3-dihydroxy benzoic acid biosynthesis by 37% and 55%, respectively above control. Likewise, glyphosate, imidacloprid, and hexaconazole decreased indole acetic acid secretion by 28%, 19%, and 34%, respectively at three times the recommended dose. Among all tested pesticides, the greatest stimulatory effect on exo-polysaccharides secretion was shown by glyphosate which stimulated Rhizobium sp. strain MRP1 to secrete exo-polysaccharides by 40% higher with respect to untreated control. Generally, the maximum toxicity to PGP traits (excluding exo-polysaccharides) of Rhizobium was shown by glyphosate, imidacloprid and hexaconazole at three times the recommended rate among herbicides, insecticides and fungicides, respectively. The results of this study implied that prior to field-application pesticides must be tested in laboratory for the adverse impact on the physiological activities of plant-beneficial soil microorganisms. This study also revealed a circumlocutory mechanism of pesticide-mediated toxicity to plant growth.

Key words: Rhizobium, Pesticide, Plant Growth Promoting Rhizobacteria (PGPR), Toxicity, Tolerance, *Pisum sativum*

Introduction

In agriculture, the pesticides are recurrently applied for three major objectives- (i) to produce a larger yield (ii) to produce crops of high quality and (iii) to reduce the input of labor and energy into crop production (Ayansina, 2009). Millions of tons of pesticides are applied annually; however, less than 5% of these products are estimated to reach the target organism, with the remainder being deposited on the soil and non-target organisms, as well as moving into the atmosphere and water (de Oliveira

et al., 2012; Ahemad and Khan, 2010a).

The surplus amount of pesticides, which does not reach the target organisms, is absorbed by the plants and hence, pesticide residues have been found in various fruits and vegetables; both raw and processed (González-Rodríguez et al., 2011). One of the most common routes of pesticide exposure in consumers is via food consumption (González-Rodríguez et al., 2008). They can have numerous negative health effects on human consumers owing to their continual exposure in the form of contaminated food products (López-Pérez et al., 2006).

The metabolic fate of pesticides is dependent on abiotic environmental conditions (temperature, moisture, soil pH, etc.), microbial community or plant species (or both), pesticide characteristics (hydrophilicity, $pK_{a/b}$, K_{ow}, etc.), and biological and chemical reactions. Abiotic degradation is due to chemical and physical transformations of the

pesticide by processes such as photolysis, hydrolysis, oxidation, reduction, and rearrangements (Van Eerd et al., 2003). Further, pesticides may be biologically unavailable because of compartmentalization, which occurs as a result of pesticide adsorption to soil and soil colloids without altering the chemical structure of the original molecule (Van Eerd et al., 2003). In soils, these excess pesticides interact with rhizosphere microorganisms including nodule bacteria (Ahemad and Khan, 2010b) and restrict the root growth in that way lead to the reduction in the number of the root sites available for the rhizobial infection (Ahemad and Khan, 2010a). Although reports of pesticidal impact on microbes are conflicting, several studies have conclusively shown that these agrochemicals are incompatible with bacterial cultures (Singh and Wright, 2002; Aamil et al., 2005).

Pesticides like metribuzin and glyphosate (herbicides); imidacloprid and thiamethoxam (insecticides); and hexaconazole, metalaxyl and kitazin (fungicides) (Table 1) are used widely in legume and non-legume crop production. However, reports of their biotoxic impacts on plant growth promoting traits (siderophores, indole acetic acid, exo-polysaccharides, hydrogen cyanide and ammonia) of pea-specific rhizobia have been very limited and are almost completely lacking in the case of the above pesticides. An attempt has, therefore, been made in the present study to determine the effects of the these pesticides on viability as well as on plant growth promoting properties of *Rhizobium* sp. strain MRP1 isolated from pea (*Pisum sativum*) nodules. It is expected that the results of this investigation would further contribute towards the more efficacious implementation of an effective pest management.

Materials and Methods
Rhizobial strains and pesticide-tolerance

A total of 50 rhizobial strains were isolated from root nodules of pea plants grown in experimental fields of Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh (27° 29' latitude and 72° 29' longitude), India using yeast extract mannitol (YEM) medium (g l^{-1}: mannitol 10; K_2HPO_4 0.5; $MgSO_4.7H_2O$ 0.2; NaCl 0.1; yeast extract 1; $CaCO_3$ 1; pH 7) (Vincent, 1970). The experimental soil was an alluvial sandy clay loam (sand 667 g kg^{-1}, silt 190 g kg^{-1}, clay143 g kg^{-1}, organic matter 6.2 g kg^{-1}, Kjeldahl N 0.75 g kg^{-1}, Olsen P 16 mg kg^{-1}, pH 7.2 and water holding capacity 0.44 ml g^{-1}, cation exchange capacity 11.7 cmol kg^{-1} and 5.1 cmol kg^{-1} anion exchange

capacity). The rhizobial strains were characterized and identified following Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and host specificity test in sterile soils (Somasegaran and Hoben, 1994). The strains were tested for their sensitivity/tolerance to chemically and functionally diverse pesticides (metribuzin, glyphosate, imidacloprid, thiamethoxam, hexaconazole, metalaxyl and kitazin) by agar plate dilution method using minimal salt agar medium (g l^{-1}: KH_2PO_4 1, K_2HPO_4 1, NH_4NO_3 1, $MgSO_4.7H_2O$ 0.2, $CaCl_2.2H_2O$ 0.02, FeSO4.7H2O 0.01, pH 6.5). The freshly prepared agar plates were amended separately with increasing concentrations of pesticides (0 to 3200 μg ml^{-1}; at a two-fold dilution interval). Later, plates were spot inoculated with 10 μl of 10^8 cells ml^{-1} rhizobial strains. Plates were incubated at 28 ±2 °C for 72 h and the highest concentration of each pesticide supporting rhizobial growth was defined as the maximum tolerance level (MTL). The experiment was replicated three times.

Quantitative assay of indole acetic acid

Indole-3-acetic acid (IAA) synthesized by rhizobial strains was quantitatively evaluated by the method of Gordon and Weber (1951), later modified by Brick et al. (1991). For this activity, the rhizobial strains were grown in Luria Bertani broth (g l^{-1}: tryptone 10; yeast extract 5; NaCl 10 and pH 7.5). Luria Bertani (LB) broth (100 ml) having fixed concentration of tryptophan (100 μg/ml) and supplemented with 0, the recommended dose (1X), two times the recommended dose (2X) and three times the recommended dose (3X) of each pesticide was inoculated with one ml culture (10^8 cells/ml) of rhizobial strains and incubated for seven days at 28±2 °C with shaking at 125 g. After seven days, a five milliliter culture from each treatment was centrifuged (9,000 g) for 15 min and an aliquot of two ml supernatant was mixed with 100 μl of orthophosphoric acid and four milliliter of Salkowsky reagent (2% 0.5M $FeCl_3$ in 35% per-chloric acid) and incubated at 28±2 °C in darkness for 1h. The absorbance of developed pink color was read at 530 nm. The IAA concentration in the supernatant was determined using a calibration curve of pure IAA as a standard.

Table 1. Pesticides used in the present study.

Category	Common name	Grade (purity)	Chemical name	Chemical family	Recommended dose	Source
Herbicides	Metribuzin	Commercial (70%w/w)	4-amino-6-*tert*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one	Triazinone	850 µg kg^{-1}	Singhal Pesticides, Mumbai, India
	Glyphosate	Commercial (71% w/w)	*N*-(phosphonomethyl)glycine	Organophosphate	1444 µg kg^{-1}	Excel Crop Core LTD., Mumbai, India
Insecticides	Imidacloprid	Technical (100% EC)	(*E*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine	Pyridylmethylamine	100 µg L^{-1}	Parijat Agrochemicals, New Delhi, India
	Thiamethoxam	Technical (100%w/w)	(*EZ*)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine	Thiazole	25 µg L^{-1}	Parijat Agrochemicals, New Delhi, India
Fungicides	Hexaconazole	Technical (100%w/w)	(*RS*)-2-(2,4-dichlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)hexan-2-ol	Conazole	40 µg kg^{-1}	Parijat Agrochemicals, New Delhi, India
	Metalaxyl	Commercial (35%w/w)	methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate	Anilide	1500 µg kg^{-1}	Tropical Agrosystem LTD., Chennai, India
	Kitazin	Commercial (48% EC)	O,O-Bis(1-methylethyl) S-phenylmethyl phosphorothioate	Organophosphate	96 µg kg^{-1}	P.I. Industries LTD., Rajasthan, India

Qualitative and quantitative estimation of siderophores

The rhizobial strains were further tested for siderophore production using Chrome Azurol S (CAS) agar medium following the method of Alexander and Zuberer (1991). Chrome Azurol S agar plates supplemented with 0, 1X, 2X and 3X of each pesticide were prepared separately and divided into equal sectors and spot inoculated with 10 μl of 10^8 cells/ml and incubated at 28±2 °C for five days. Development of yellow orange halo around the bacterial growth was considered as positive test for siderophores-biosynthesis. The production of siderophores by the test strains were further detected quantitatively using Modi medium (K_2HPO_4 0.05%; $MgSO_4$ 0.04%; NaCl 0.01%; mannitol 1%; glutamine 0.1%; NH_4NO_3 0.1%) (Reeves et al., 1983). Modi medium amended with 0, X, 2X and 3X of each pesticide, was inoculated with 10^8 cells ml^{-1} of bacterial cultures and incubated at 28±2 °C for five days. Catechol type phenolates were measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride-ferricyanide reagent of Hathway. Ethyl acetate extracts was prepared by extracting 20 ml of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent was prepared by adding one milliliter of 0.1 M ferric chloride in 0.1 N HCl to 100 ml of distilled water, and to this, was added one milliliter of 0.1 M potassium ferricyanide (Reeves et al., 1983). For the assay, one volume of the reagent was added to one volume of sample and absorbance was determined at 560 nm for salicylic acid (SA) with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2, 3- dihydroxy benzoic acid (DHBA) as standard.

Assay of hydrogen cyanide (HCN), ammonia and exo-polysaccharides

Hydrogen cyanide production by rhizobial strains was detected by the method of Bakker and Schipper (1987). For HCN production, all rhizobial strains were grown on an HCN induction medium (g l^{-1}: tryptic soy broth 30; glycine 4.4; agar 15) supplemented with 0, 1X, 2X and 3X of each pesticide at 28±2 °C for four days. Further, 100 μl of 10^8 cells/ml of each rhizobial strain was placed in the centre of the petri plates. A disk of Whatman filter paper No. 1 dipped in 0.5% picric acid and 2% Na_2CO_3 was placed at the lid of the petri plates. Plates were sealed with parafilm. After four days incubation at 28±2 °C, an orange brown color of the paper indicating HCN production was observed.

For ammonia assessment, the rhizobial strains were grown in peptone water with 0, 1X, 2X and 3X of each pesticide and incubated at 28±2 °C for four days. One milliliter of Nessler reagent was added to each tube and the development of yellow color indicating ammonia production was recorded following the method of Dye (1962).

The exo-polysaccharide (EPS) produced by the rhizobial strains was determined as suggested by Mody et al. (1989). For this, the bacterial strains were grown in 100 ml capacity flasks containing basal medium supplemented with 5% sucrose and treated with 0, 1X, 2X and 3X of each pesticide. Inoculated flasks were incubated for five days at 28±2°C on rotary shaker (100 g). Culture broth was spun (5433 g) for 30 min and EPS was extracted by adding three volumes of chilled acetone (CH_3COCH_3) to one volume of supernatant. The precipitated EPS was repeatedly washed three times alternately with distilled water and acetone, transferred to a filter paper and weighed after overnight drying at room temperature. Each individual experiment was repeated three times.

Statistical Analysis

The experiments were conducted in three replicates using the same treatments. The difference among treatment means was compared by high range statistical domain (HSD) using Tukey test at 5% probability level on SPSS 10 software.

Results

Characterization, identification and pesticide-tolerance

In the present study, a total of 50 rhizobial strains recovered from nodules of pea root systems were identified on the basis of morphological and biochemical tests and host-specificity test for nodulation in sterile soils and monitored further for pesticide-tolerate by exposing them to the graded concentrations of herbicides (metribuzin and glyphosate), insecticides (imidacloprid and thiamethoxam) and fungicides (hexaconazole, metalaxyl and kitazin) (Table 1) on minimal salt agar medium. Among these strains, *Rhizobium* sp. strain MRP1 was specifically selected due to the highest MTL for all selected herbicides, insecticides and fungicides (Figure 1, Table 2) and the maximum production of PGP substances (siderophores, IAA, EPS, HCN and ammonia) (Table 3).

Siderophore production

Production of siderophores by the pesticide-tolerant *Rhizobium* sp. strain MRP1 was determined on CAS agar plates supplemented with varying concentrations of pesticides. *Rhizobium* sp. strain MRP1 displayed siderophores-producing potential

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by forming an orange zone of 11 mm size on pesticide free CAS agar medium.

Table 2. Morphological and biochemical characteristics of *Rhizobium* sp. strain MRP1.

Characteristics	Strain MRP1
Morphology	
Gram reaction	-
Shape	rods
Biochemical reactions	
Citrate utilization	-
Indole	+
Methyl red	+
Nitrate reduction	+
Oxidase	-
Voges Proskaur	+
Carbohydrate utilization	
Dextrose	-
Lactose	-
Mannitol	+
Sucrose	-
Hydrolysis	
Starch	+
Gelatin	-
Maximum tolerance level (MTL) to	
Metribuzin	3000 μg ml^{-1}
Glyphosate	2800 μg ml^{-1}
Imidacloprid	1600 μg ml^{-1}
Thiamethoxam	2200 μg ml^{-1}
Hexaconazole	2000 μg ml^{-1}
Metalaxyl	2600 μg ml^{-1}
Kitazin	3000 μg ml^{-1}

+ indicates positive and – indicates negative reactions

In general, addition of pesticides at the recommended field rates to the medium did not reduced the siderophore-zone formed by pure culture of *Rhizobium* sp. strain MRP1. At three times the recommended rates, the effect of imidacloprid, hexaconazole and metalaxyl was inhibitory to siderophore-zone. For example, fungicide hexaconazole reduced the siderophore-zone to the highest degree by 18% over untreated control. In addition, the degree of zone-inhibition was not co-related (R^2 = 0.009) with the concentration of each pesticide (Table 3).

Furthermore, the ethyl acetate extraction from culture supernatant of *Rhizobium* sp. strain MRP1 grown in the Modi medium devoid of pesticides yielded 32 and 22 μg/ml SA and DHBA type siderophores. Pesticide-concentration dependent progressive decline for both siderophore molecules was observed. Nevertheless, degree of pesticide-mediated decrease for SA and DHBA differed from the type and functional group of each pesticide. Within herbicide group, glyphosate showed the highest toxicity to the synthesis of SA and DHBA.

For instance, glyphosate at 3X decreased SA and DHBA secretion by 19% and 32%, respectively compared to control. Among insecticides, thiamethoxam at 3X showed the most deleterious effect on the SA production while both imidacloprid and thiamethoxam at 3X most adversely affected the DHBA synthesis. For instance, three times the recommended dose of thiamethoxam decreased SA by 28% whereas three times the recommended dose of both imidacloprid and thiamethoxam decreased the DHBA secretion by 27% relative to control. Among fungicides, hexaconazole at three times the recommended dose triggered the maximum stress on siderophore-biosynthesis by *Rhizobium* sp. strain MRP1 and decreased SA and DHBA by 37% and 55%, respectively above control. Among all pesticides, hexaconazole at 3X in general, displayed the most toxic effect on SA and DHBA synthesis.

Indole acetic acid production

The effect of the three concentrations of each pesticide on IAA synthesized by *Rhizobium* sp. strain MRP1 varied considerably. In the absence of pesticides, *Rhizobium* sp. strain MRP1 produced a maximum (32 μg/ml) amount of IAA. In contrast, the amount of IAA released by *Rhizobium* sp. strain MRP1, however, decreased progressively with the graded addition of each pesticide in LB broth. Of herbicides, insecticides and fungicides, most severe effect on IAA synthesis was evident in the presence of glyphosate, imidacloprid and hexaconazole, respectively. For example, glyphosate decreased IAA by 12%, 22% and 28%, imidacloprid by 9%, 12% and 19% and hexaconazole by 12%, 22% and 34% at 1X, 2X and 3X, respectively. On comparing the toxicity of specific concentration of each pesticide, hexaconazole in general had the most toxic impact on IAA bio-synthesis by *Rhizobium* sp. strain MRP1 (Table 3).

Production of exo-polysaccharides, HCN and ammonia

Unlike other PGP substances produced by *Rhizobium* sp. strain MRP1 exposed to pesticide-stress, the amount of EPS synthesized increased progressively with gradual increment of each pesticide in culture medium. Among all tested pesticides, the greatest stimulatory effect on EPS secretion was shown by glyphosate which stimulated *Rhizobium* sp. strain MRP1 to secrete EPS by 40% higher with respect to untreated control (Table 3). Interestingly, the three concentrations of each herbicide, insecticide and fungicide did not affect negatively HCN and ammonia synthesis by *Rhizobium* sp. strain MRP1 (Table 3).
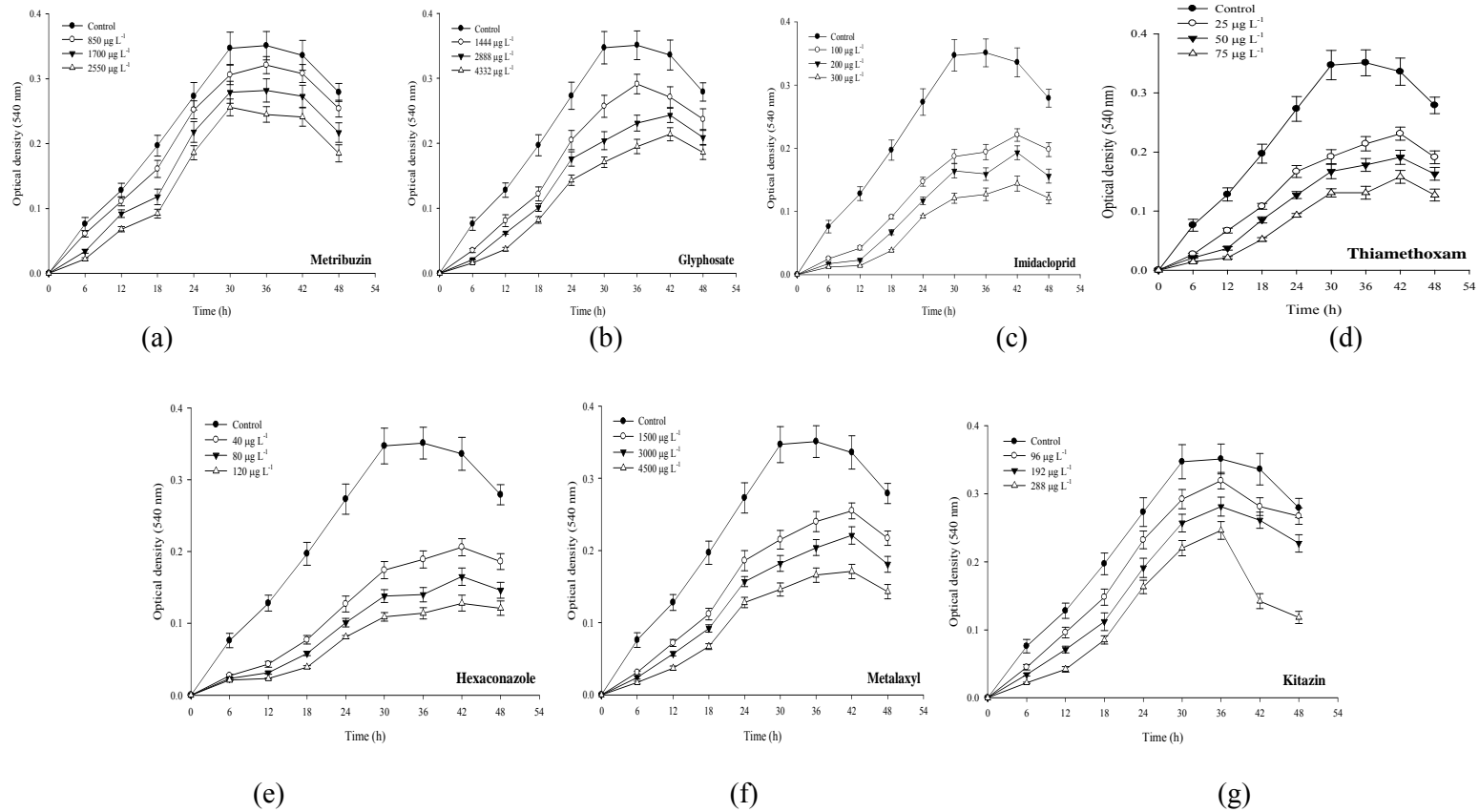
Figure 1. Impact of the recommended (*open circle*), double (*filled inverted triangle*) and three times (*open upright triangle*) the recommended rates of metribuzin (a), glyphosate (b), imidacloprid (c), thiamethoxam (d), hexaconazole (e), metalaxyl (f) and kitazin (g) on *Rhizobium* strain MRP1 (in terms of optical density) grown in minimal salt agar medium.

Table 3. Plant growth promoting activities of *Rhizobium* strain MRP1 in the presence of varying concentrations of pesticides.

Pesticides	Dose rate ($\mu g\ l^{-1}$)	Plant growth promoting activities						
		Siderophores			IAA[d] ($\mu g\ ml^{-1}$) 100T[e]	EPS[f] ($\mu g\ ml^{-1}$)	Ammonia	HCN[g]
		Zone on CAS[a] agar (mm)	SA[b] ($\mu g\ ml^{-1}$)	DHBA[c] ($\mu g\ ml^{-1}$)				
Metribuzin	850	11±2a	31±1.5ab	20±1.2b	30±1.8ab	22±2.1de	+	+
	1700	11±2a	29±1.6b	18±1.7d	27±1.4de	24±1.3bc	+	+
	2550	11±1a	27±1.3cd	16±1.6f	26±2.1e	27±2.5ab	+	+
Glyphosate	1444	11±2a	30±1.7ab	19±1.3cd	28±1.8cd	22±1.7de	+	+
	2888	11±1a	28±1.3b	17±1.3ef	25±1.9f	25±2.4bc	+	+
	4332	11±1a	26±1.2d	15±1.2gh	23±1.4g	28±1.2a	+	+
Imidacloprid	100	11±2a	28±1.4b	20±1.2b	29±1.3bc	21±1.2ef	+	+
	200	10±2ab	26±1.3d	18±1.1d	28±1.6cd	24±2.3bc	+	+
	300	10±1ab	24±1.2f	16±1.5f	26±1.7e	26±2.4ab	+	+
Thiamethoxam	25	11±2a	30±1.1ab	21±1.3ab	31±2.3ab	20±2.3f	+	+
	50	11±1a	28±1.2b	18±1.7d	30±2.3ab	21±2.2ef	+	+
	75	11±1a	23±1.6g	16±1.2f	28±1.5cd	23±1.1cd	+	+
Hexaconazole	40	11±2a	26±1.3d	14±1.5h	28±2.4cd	22±1.2de	+	+
	80	10±1ab	23±1.5g	12±1.3i	25±1.6f	24±2.5bc	+	+
	120	9±1b	20±1.1h	10±1.5j	21±1.4h	25±1.6bc	+	+
Metalaxyl	1500	11±1a	28±1.5b	19±1.2cd	28±1.7cd	22±1.9de	+	+
	3000	11±2a	27±1.6cd	18±1.5d	26±2.3e	23±2.2cd	+	+
	4500	10±1ab	25±1.2ef	16±1.3f	23±1.9g	25±2.2bc	+	+
Kitazin	96	11±1a	29±1.7b	20±1.2b	30±1.8ab	20±1.5fg	+	+
	192	11±1a	28±1.3b	19±1.5cd	27±1.6de	21±2.2ef	+	+
	288	11±1a	26±1.0d	17±1.5ef	25±2.1f	23±1.8cd	+	+
Control (without pesticide)		11±1a	32±1.5a	22±1.1a	32±1.6a	20±1.3f	+	+
LSD (p ≤ 0.05)		0.67	2.41	1.35	2.31	1.42	-	-
F value (treatment)		74.2	238.4	155.9	278.6	384.7	-	-

Values indicate mean of three replicates. Mean values (±S.D.) followed by different letters are significantly different within a row or column at p ≤ 0.05 according to Tukey test. aChrome azurol S agar; bSalicylic acid; c2,3 Dihydroxy benzoic acid; dIndole acetic acid; eTryptophan concentration ($\mu g\ ml-1$); fExopolysaccharides; gHydrogen cyanide; + indicates positive reaction

Discussion
Pesticide-tolerance
In our study, *Rhizobium* sp. strain MRP1 depicted the abnormally high tolerance to an array of the selected pesticides of various chemical families. The MTL values of pesticides ranged from 2200 $\mu g\ ml^{-1}$ to 3000 $\mu g\ ml^{-1}$. Tolerance or resistance in microorganisms against pesticides is a complex process which is regulated both at physiological/genetic level of microorganism. And hence, the microorganisms that developed resistance to pesticides are frequently capable of biodegrading them (Kumar et al., 1996; Ortiz-Hernández and Sánchez-Salinas, 2010). The temporary resistance (tolerance) against pesticides in general, is attributed to physiological changes that induce the microbial metabolism for the formation of a new metabolic pathway to bypass a biochemical reaction inhibited by a specific

pesticide (Bellinaso et al., 2003). Permanent resistance, on the other hand, depends upon genetic modifications, inherited by the subsequent generation of microbes (Johnsen et al., 2001; Herman et al., 2005).

Siderophore production
In the present study, *Rhizobium* sp. strain MRP1 exhibited plant growth promoting traits like production of siderophores, phytohormone and exopolysaccharides in substantial amount in both the absence and presence of pesticide-stress. In the aerobic environment, iron occurs principally as Fe^{3+} and is likely to form insoluble hydroxides and oxyhydroxides, thus making it generally inaccessible to microorganisms. To acquire sufficient iron, the most commonly found strategy in bacteria is the secretion of siderophores, low-molecular mass iron chelators with high association constants for complexing iron. Thus, siderophores

act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation (Miethke and Marahiel, 2007; Khan et al., 2010).

Indole acetic acid production

In our study, *Rhizobium* sp. strain MRP1 produced IAA in substantial amount both in the presence and the absence of pesticide-stress. Plant hormones play an important role for the growth and development of plants. Auxins like indole-3-acetic acid (IAA) are among the most studied growth regulators and are believed to be essential for plants because no plants are known to be unable to synthesize it (Callis, 2005). It is the most common auxin in plants. Other compounds with auxin-like activity, such as indole-3-butyric acid, phenyl acetic acid, and 4-chloro-IAA, also reported, but little is known about their physiological roles (Kende and Zeevaart, 1997). Although the ability to produce phytohormones is primarily attributed to the plant kingdom, they are also widespread among soil and plant associated microbes such as bacteria including *Rhizobium* sp. (Costacurta and Vanderleyden, 1995). Phytohormones produced by microbes in the rhizosphere are a means for their interaction with plants (Christiansen-Weniger, 1998). By the excretion of IAA synthesized from transamination and decarboxylation of tryptophan, these microbes locally change the endogenous hormone balance of the host, thereby promoting plant cell division, growth, and nutrient release, and supporting their own growth (Glick et al., 1999; Khan et al., 2010). Moreover, a low level of IAA produced by rhizosphere bacteria promotes primary root elongation whereas a high level of IAA stimulates lateral and adventitious root formation but inhibit primary root growth (Ma et al., 2009).

Production of exo-polysaccharides, HCN and ammonia

Rhizobium sp. strain MRP1 produced other PGP substances like EPS, HCN and ammonia. The EPS helps to protect bacteria against desiccation, phagocytosis and phage attack besides supporting N_2 fixation by preventing high oxygen tension (Tank and Saraf, 2003). Moreover, EPS play a very important role in legume-*Rhizobium* symbiosis, as acidic EPS produced by rhizobia are essentially required for nodule invasion and, consequently, for effective nitrogen-fixing symbiosis with many legumes in nodule formation (Ahemad and Khan, 2012).

Rhizobacteria protect the growing plants from pathogen attack by directly killing parasites by producing HCN (Devi et al., 2007). The ammonia released by the rhizobacterial strain plays a signaling role in the interaction between rhizobacteria and plants and also increase the glutamine synthetase activity (Chitra et al., 2002).

Decline of PGP substances released by Rhizobium exposed to pesticide-stress

Each PGP trait of bacteria is the result of sequential metabolic reactions mediated by various specific functional proteins (enzymes) along the defined metabolic pathway. The metabolic pathways for any specific PGP trait may be more than one depending upon the type of the PGP substances and bacterial genera/species (Ahemad and Khan, 2011a). Pesticides adversely affect protein synthesis and the metabolic enzymes (Kapoor and Arora, 1996; Boldt and Jacobsen, 1998). Therefore, it seems probable that pesticides employed in this study might have inhibited the functioning of the enzymes participating in different metabolic pathways of PGP traits in *Rhizobium* sp. strain MRP1. Additionally, pesticides not only damage structural proteins essential for growth of the organism but also responsible for geno-toxicity (Pham et al., 2004) and eventually leads to the decreased functioning and survival of organisms exposed to high concentration of pesticides (Kumar et al., 2010; Ahemad and Khan, 2011b).

Conclusions

Overall, this study implies that pesticides not only affect the growth of pea-specific rhizobia but also have an adverse impact on their PGP activities. This study suggested that screening of pesticides on the basis of degree of *in vitro* toxicity to PGP functions of beneficial rhizobacteria would result into eco-friendly pest management as well as sustainability of soil fertility. These results imply further research on toxicological effects of pesticides on PGP activities of soil microflora at molecular level to fortify the effective implementation of this approach to protect the soil ecosystem from pesticide hazard.

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PLANT SCIENCE

Antibacterial activity of organic extracts from *Zinnia peruviana* (L.) against gram-positive and gram-negative bacteria

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Abstract

Latin American countries have a long tradition in the use of plants that can produce varied therapeutic effects. *Zinnia peruviana* (L.) (Asteraceae) is a native plant used in folk medicine for the treatment of malaria, for stomach pain, as hepatoprotective and antiparasitic, antifungal and antibacterial agents. The antibacterial activity *in vitro* of extracts of *Z. peruviana* was evaluated against methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74910, *Escherichia coli* and *Bacillus cereus*. Different extracts were prepared using ethyl acetate and mixtures of n-hexane and ethyl acetate of increasing polarity on flash chromatography. *Z. peruviana* extracts showed antibacterial effects against all gram-positive and gram-negative pathogenic bacteria tested, with significant antibacterial activity against methicillin-resistant *S. aureus*, *L. monocytogenes* and *B. cereus*. The results open a path for future studies in the search for new molecules of natural origin with antibiotic activity.

Key words: Antibacterial activity, Organic extracts, *Zinnia peruviana*

Introduction

The production of drugs for the pharmacological treatment of diseases began with the use of herbs. Latin American countries have a long tradition in the use of plants which contain compounds with biological activity that can produce therapeutic effects varied (Goleniowski et al., 2006). Microbial resistance to different antibiotics used today, has caused a trend to search for drugs of natural origin.

Zinnia peruviana (L.) (Asteraceae) is a native plant known by the vernacular names "Chinita del Campo". This specie is found in the center and north of Argentina (Barrie et al., 2011; Cantero et al., 2000). It is an erect herbaceous annual, 70-100 cm in height, with lance linear to broadly ovate or elliptic leaves, and 4-5 cm wide capitula with ray florets in a single whorl. Ligules are either yellow or scarlet red (Stimart et al., 2007). Nowadays,

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there is not much information of the bioactivity of different organic extracts of this plant. However, this specie is widely used in folk medicine for the treatment of malaria (Carrizo et al., 2002; Del Vitto et al., 1997; Goleniowski et al., 2006), for stomach pain, as hepatoprotective and antiparasitic (Salgado, 2007), antifungal and antibacterial agents (Barboza et al., 2009). The purpose of the study presented here, was to evaluate *in vitro* the antibacterial activity of organic extracts of *Z. peruviana*.

Materials and methods
Plant material

Zinnia peruviana (L.) (Asteraceae) aerial parts were collected in Rio Grande, San Luis, Argentina in February 2008 (Figure 1). Voucher specimen was identified by Ing Luis Del Vitto *et al*. and lodged in the University of San Luis (Argentina) herbarium.

Preparation of extracts

Previously dried aerial parts (500g) at room temperature and finely powdered were macerated with acetone at room temperature for 48h. Acetone extract was separated by filtration. Extraction was replicated three times. Extraction fluids were concentrated under reduced pressure yielding 330 g of dark syrup, then; it was dissolved with acetone and absorbed on silica gel column (700g). Each acetone extract was partitioned by chromatography "Flash" using as elution solvents ethyl acetate

(AcOEt) and mixtures of n-hexane and AcOEt of increasing polarity. The progress of separation was monitored by thin layer chromatography (TLC) using as mobile phase benzene: dioxane: acetic acid (120:20:4) and as revealing a mixture of H_2SO: AcOH: H_2O (2:20:1) followed by heating at 120°C. The extracts of *Z. peruviana* tested in this study were: 100% ethyl acetate, 30% ethyl acetate/n-hexane and 40% ethyl acetate/n-hexane.

Microorganism

A total of five bacteria were selected for this study. Methicillin-resistant *Staphylococcus aureus* ATCC 43300, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* CLIP 74902 (Collection Listeria Institute Pasteur), *Escherichia coli* and *Bacillus cereus* isolated in UNSL Laboratory.

Determination of Minimal Inhibitory Concentration (MIC)

The antibacterial activity was assayed *in vitro* using microplate method (*microwell dilution)* according to the CLSI method (Wilkinson, 2007) in tripticase soya broth (Britania, Argentina) pH7.2 supplemented with 0,01% (w/v) of 2,3,5-triphenyltetrazolium chloride (TTC) used as visual indicator of bacterial growth.

The inoculum of each strain was prepared from 24h broth culture and adjusted to concentration of 10^6CFU/ml. Organic extracts were dissolved in dimethylsulfoxide and tested in a concentration ranging from 8 to 0.1 mg/ml.

The 96-well plates were prepared by dispensing into each well 95µl of nutrient broth and 5 µl of the inoculum (final concentration of 10^4 CFU/ml). One hundred microlitre aliquot from the serial dilutions of extracts was transferred into consecutive wells. The final volume in each well was 200 µl. Controls of nutrient broth, strains and extracts were included. After 24- hour incubation at 37ºC, the antibacterial activity of the extracts (MIC) was defined as the lowest concentration of the extract in the medium in which there no visible grown. The experiments were replicated at least twice.

Determination of minimal bactericidal concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the tripticase soya agar plates, in order to evaluate bacterial growth. MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after 24 h of aerobic incubation at 37°C.

Results and Discussion

Argentina is a country with both rich floral biodiversity and cultural diversity. Traditional herbal medicines are important in the health care of most people, and rely heavily on the use of indigenous plants (Landa et al., 2007).

The increasing prevalence of multidrug resistant bacteria and the recent appearance of strains with reduced susceptibility to antibiotics raises the specter of untreatable bacterial infections and adds urgency to the search for new infection-fighting strategies (Sieradski et al., 1999). In the present study, the extracts of *Z. peruviana* showed antibacterial effects against all gram-positive and gram-negative pathogenic bacteria tested (Table1). Although, Gram positive bacteria showed the highest sensitivity against the tested extracts. *Z. peruviana* 30% and 40% ethyl acetate/n-hexane extracts showed a similar behavior, with significant antibacterial activity against methicillin-resistant *S. aureus, L. monocytogenes* and *B. cereus* CIM of 0.2 mg/ml. An interesting finding was that all the tested extracts showed activity against methicillin-resistant *S. aureus*. This organism is highly infectious and often is resistant to multiple drugs which making it very difficult the therapeutic options (Drew, 2007). Furthermore, we observed that *P. aeruginosa,* nosocomial opportunistic microorganism which is characterized by presenting multiple mechanisms of antimicrobial resistance and to produce serious infections in hosts with altered defenses, was sensitive to all the extracts tested at a concentration of 4 mg/ ml. *E. coli* showed MIC of 8 mg/ml for *Z. peruviana* 30% and 40% ethyl acetate/n-hexane extracts (Figure 2). Moreover, *B. cereus* and *L. monocytogenes* pathogenic bacteria that cause foodborne infections serious were strongly inhibited by those extracts (MIC 0.2 mg/ml). *Z. peruviana* 100% ethyl acetate extract, showed significant activities against all tested strains at doses of 4 mg/ml. The MBC values were identical to the MIC values or two fold higher than the corresponding MIC (Table 1).

There are few reports on the antimicrobial activity of this plant. Some authors demostrated that aqueous and alcoholic extracts from *Z. peruviana* showed different degrees of antibacterial activity against gram-positive and gram-negative microorganisms such as *Enterococcus faecalis*, *E.coli*, *Klebsiella pneumoniae*, *P. aeuruginosa, Serratia marcescens, S. aureus, Streptococcus agalactiae*, *Streptococcus pyogenes* (Amani et al., 1998).

Table 1. Antibacterial activity of *Z.peruviana* extracts.

Bacterial strains	Extracts MIC/MBC (mg/ml)		
	100% ethyl acetate	30% ethyl acetate/n-hexane	40% ethyl acetate/n-hexane
S. aureus ATCC 43300	4/4	0.2/0.4	0.2/0.4
P. aeruginosa	4/10	4/8	4/8
L. monocytogenes CLIP 74902	4/8	0.2/0.4	0.2/0.2
E. coli	4/8	8/8	8/8
B. cereus	4/8	0.2/0.4	0.2/0.4

MIC: Minimal Inhibitory Concentration (mg/ml); MBC: Minimal Bactericidal Concentration (mg/ml).

Figure 1. *Zinnia peruviana* (L.) (Asteraceae) collected in Rio Grande, San Luis (Argentina). February 2008.
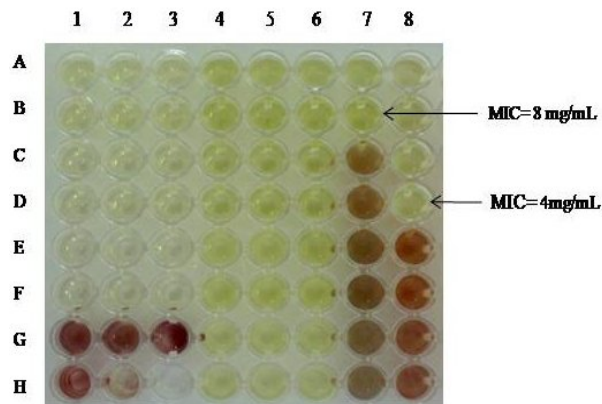

Figure 2. Microdilution plate used for broth microdilution method with *Z. peruviana* 30% ethyl acetate/n-hexane extract against *Staphylococcus aureus* ATCC 43300 (4), *Listeria monocytogenes* CLIP 74902 (5), *Bacillus* cereus (6), *Escherichia coli* (7) and *Pseudomonas aeruginosa* ATCC 27853 (8).
File 1: A-F: broth controls, G and G: controls of strains. Files 2 and 3, A-F extract controls. G and H: controls of strains.

To our knowledge, there are no reports available in the literature on the phytochemical screening of *Z. peruviana*. Some authors detected compounds such as saponins, steroid, flavonoids and glycosides from *Zinnia elegans* (Ehsanulhaq, 2001; Hafizaet et al., 2002). Several authors have demonstrated that these compounds obtained of different medicinal plants inhibited the growth of gram positive and gram negative bacteria (Aslam et al., 2009; Gurinder et al., 2009; Sarojini et al., 2011). So, if some of these phytoconstituents are present in *Z. peruviana,* they could be responsible, partly or completely, of antimicrobial activity observed here from organic extracts of this plant.

Conclusions

This study contributes to knowledge of the antibacterial properties of regional plant extracts and opens a path for future studies in the search for new molecules of natural origin with antibiotic activity.

Acknowledgement

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ANIMAL SCIENCE

Evaluation of nutritive values of tropical feed sources and by-products using *in vitro* gas production technique in ruminant animals

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Abstract

Nutritive value and fermentation characteristics of beans pods (BPS), dussa (fermented sorghum wastes) (DSS), groundnut shells (GNS) and maize offal (MZO) were evaluated by measuring the gas production in vitro for a period of 96 h. The crude protein (CP) contents were 12.24, 2.17, 7.39 and 2.54 % for BPS, DSS, GNS and MZO respectively. MZO showed the highest level of gas cumulative gas production all levels of incubation. The NDF, ADF and ADL were significantly different ($p < 0.05$) among the agricultural wastes used in this study. The BPS, GNS and MZO showed the highest levels of NDF and ADF. The cumulative gas production for GNS was significantly ($p < 0.05$) lower in comparison with other agricultural wastes. The fractional fermentation rate (c) at different times of incubation was high for DSS, BPS and MZO and lowest for GNS. Fermentation of the insoluble fraction (b) followed the same pattern. The short chain fatty acid (SCFA) ranged from 0.370 to 0.695 μm while organic matter digestibility (OMD) ranged from 39.27 to 49.63 %. Beans pod exhibited the greatest estimated Metabolisable Energy (ME), SCFA and OMD. This result suggests that the tropical feed sources under study are all potential sources of energy for ruminant animals.

Key words: In-vitro gas production, Nutritive value, Ruminant, Tropical feeds

Introduction

The use of crop residues and agricultural by-products in animal feeding is a very common practice in tropical countries especially Nigeria. Evaluating the nutritive value of these available feed resources are important as these could make an important contribution to the nutrition of livestock (Taphizadeh et al., 2008).

Fermentation characteristics of feedstuffs in buffered rumen fluid can be studied using *in vitro* techniques (Cone et al., 1997). The *in vitro* gas production system helps to better quantify nutrient utilization and its accuracy in describing digestibility in animals has been validated in numerous experiments (Taphizadeh et al., 2008).

In vitro gas production techniques stimulate the rumen fermentation process and they have been used to evaluate the potential of feed to supply nutrients to ruminants (Sandoval Castro et al.,

2003).

There is therefore a need to develop, for use in tropical countries especially in Nigeria where simple and cheap techniques, can be used to screen rapidly agricultural wastes and by-products. In view of these, *in vitro* gas production readily comes to mind and could play an important role.

Materials and Methods
Sample collection

Dried samples of agricultural wastes (beans pods (BPS), dussa (fermented sorghum wastes) (DSS), and groundnut shells (GNS) and maize offal (MZO) were collected from the Teaching and Research Farm, Nasarawa State University, Shabu-Lafia, Nigeria. The samples were mill through a 1 mm screen and oven-treated at 65°C until a constant weight was obtained for dry matter determination.

Chemical analysis

Nitrogen (N) content of the agricultural wastes was determined by the standard Kjeldhal method (AOAC, 1991) and the amount of crude protein was calculated (Nx6.25). Neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), and crude fibre (CF) were assessed using the methods proposed by Van Soest et al, (1991).

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Concentrations of Ca, Mg and K of feedstuffs were determined by atomic absorptions spectrophotometer (GBC 908AA, GBA Australia).

In vitro gas production study

Rumen fluid was obtained from three West African Dwarf female goats. The method of collection was as described by Babayemi and Bamikole (2006a) using suction tube from goats previously fed with 40% concentrate feed (40% corn, 10% wheat offal, 10% palm kernel cake, 20% groundnut cake, 5% soybean meal, 10% dried brewers grain, 1% common salt, 3.75% oyster shell and 0.25% fish meal) and 60% *Pannicum maximum* at 5% body weight. The rumen liquor was collected into the thermo flask that had been pre warmed to a temperature of $39^{o}C$ from the goats before they were offered the morning feed. Incubation procedure was as reported by Menke and Steingass (1988) using 120 ml calibrated transparent plastic syringes with fitted silicon tube. The sample weighing 200 mg (n=3) was carefully dropped into syringes and thereafter, 30 ml inoculums containing cheese cloth strained rumen liquor and buffer (g/litre) of 9.8 $NaHCO_3$ + 2.77 Na_2HPO_4 + 0.57 KCl + 0.47 $NaCl$ + 2.16 $MgSO_3$ $7H2O$ + 16 $Cacl2$. $2H_2O$) (1:4 v/v) under continuous flushing with CO_2 was dispensed using another 50 ml plastic calibrated syringe. The syringe was tapped and pushed upward by the piston in order to completely eliminate air in the inoculums. The silicon tube in the syringe was then tightened by a metal clip so as to prevent escape of gas. Incubation was carried out at $39\pm1^{o}C$ and the volume of gas production was measured at 3, 6, 9, 12, 15, 18, 21, 24, 48, 72 and 96 h, although the gas production is always critical between 21-96h. At post incubation period, 4 ml of NaOH (10M) was introduced to estimate the methane production as reported by Fievez et al., (2005). The post incubation parameters such as metabolisable energy, organic matter digestibility and short chain fatty acids were estimated at 24 h post gas collection according to Menke and Steingass (1988). The average of the volume of gas produced from the blanks was deducted from the volume of gas produce per sample against the incubation time and from the graph, the gas production characteristics were estimated using the equation $Y = a+b\ (1-e^{-ct})$ as described by Orskov and McDonald (1979). Where Y = volume of gas produced at time t, c = intercept (gas produced from the insoluble fraction (b), t= incubation time. Metabolisable energy (ME) was calculated as ME = $2.20 + 0.136Gv + 0.057CP + 0.0029\ CF$ (Menke and Steingass, 1988), organic matter digestibility (OMD) (%) was assessed as OMD = $14.88 + 889Gv + 0.45CP + 0.651XA$ (Menke and Steingass, 1988). Short chain fatty acids (SCFA) as $0.0239\ V – 0.0601$ (Getachew et al.,1999) where Gv, CP CF and XA are total gas volume, crude protein, crude fibre and ash, respectively.

Statistical Analysis

Data obtained were subjected to analysis of variance of SAS, (1998). Where significant differences (p<0.05) occurred, the means were separated using Duncan's multiple range test.

Results and Discussion

The chemical composition of the evaluated agricultural wastes and by-products is presented in Table 1. The result shows that there were wide variations in crude protein (CP), crude fibre (CF), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) content. The CP content of beans pods (BPS) was higher than that of other feeds under study with the least CP obtained from dussa (fermented sorghum wastes) (DSS).

Table 1. Chemical composition (g/100g DM) of different Agricultural waste.

Parameters	BPS	DSS	GNS	MZO	±SEM
Dry Matter	90.83[b]	90.52[b]	92.17[a]	90.61b	0.10
Crude protein	12.25[a]	2.17[c]	7.39[b]	2.54c	0.28
Ether extract	6.13	5.4	6.31	5.85	0.18
Ash	7.64[a]	6.39[b]	7.79[a]	7.53a	0.11
Crude fibre	30.79[a]	23.47[d]	26.15[c]	28.17b	0.21
Nitrogen Free Extract	50.79[b]	53.00[a]	44.53[d]	46.18c	0.20
Neutral Detergent fibre	71.44[a]	68.59[c]	69.41[b]	69.68b	0.13
Acid Detergent lignin	17.15[a]	13.96[c]	15.31[b]	14.85b	0.13
Acid Detergent fibre	52.35[a]	46.92[c]	51.08[b]	51.74b	0.12
Cellulose	35.13[b]	33.15[c]	35.77[ab]	36.89[a]	0.23
Hemicellulose	19.06[b]	21.33[a]	17.33[d]	17.94[a]	0.10

a,b,c,d means on the same column with different superscripts are significantly varied (P < 0.05), BPS = cowpea pod, DSS =dussa, GNS= groundnut shells, MZO= maize offal, SEM= standard error of the mean.

Table 2. Mineral composition (mg/Kg) of major minerals and trace minerals (ppm) of agricultural waste.

Minerals	BPS	DSS	GNS	MZO	+SEM
Major minerals					
Calcium	7.36[a]	3.55[d]	6.44[b]	4.16[c]	0.03
Phosphorus	0.91	0.727	1.343	0.72	0.12
Magnesium	5.345[a]	1.133[c]	2.53[b]	0.824[d]	0.07
Sodium	0.514[a]	0.054[c]	0.04[c]	0.268[b]	0.07
Potassium	0.346[c]	0.165[c]	7.82[a]	0.824[b]	0.01
Trace minerals					
Iron	4.98a	2.21b	0.129c	0.189c	0.12
Copper	0.0201	0.0274	0.0195	0.0107	0.00
Zinc	0.0586c	0.106c	0.0587c	0.0746b	0.00
Manganese	0.161b	0.033c	0.234a	0.083c	0.01

a,b,c,d means on the same column with different superscripts are significantly varied (P < 0.05), BPS = cowpea pod, DSS =dussa, GNS= groundnut shells, MZO= maize offal, SEM= standard error of the mean.

The CF content differed significantly (p<0.05) among the feedstuffs, whereas there were no significant difference (p>0.05) in the ether extract (EE) content. The NDF ranged from 69.41 to 71.44%, ADF from 46.92 to 52.35% and ADL from 13.96 to 17.15%. The beans pods (BPS), dussa (DSS), and maize offal (MZO) showed the lower DM contents while groundnut shell had the highest DM. All the obtained mineral content (Table 2) with the exception of phosphorus and copper differed significantly (p<0.05).

Wide variations were also observed in the gas volume production at different hours of incubation (Table 3). The result indicates that the cumulative gas volume after 24, 48, 72 and 96h of incubation was significantly different (p< 0.05). The gas volumes at 96 h ranked from the highest to the lowest: MZO, BPS and GNS respectively.

The fermentation of the insoluble fraction (b) of, BPS DSS and MZO were: 35.33, 41.33, 34.33 and 37.00mL respectively.

In vitro OMD, SCFA, ME and methane at 24h of incubation are shown in Table 4. The estimated ME, SCFA, OMD and CH_4 significantly differ (p<0.05) among the tested feedstuffs.

The variation observed in the chemical composition and mineral content of the different feedstuffs could be due to many factors such as stage of growth, maturity, species or variety (Von Keyserlingk et al., 1996; Agbagla-Dohanni et al., 2001; Promkot and Wanapat, 2004), drying method, growth environment (Mupangwa et al., 1997) and soil types (Thu and Preston, 1997). These listed factors may partially explain the differences in chemical composition (Chumpuwadee et al., 2007) between this study and others.

It can be seen that the fermentation of the insoluble fraction (b) of beans pod (BPS) and groundnut shell were low when compared to other feeds, probably a reflection of high level of lignin (Chumpuwadee et al., 2005). Additionally, cowpea pod (BPS) and groundnut shells (GNS) had high protein content. The protein fermentation does not lead to extensive gas production (Khazaal et al., 1995). The higher fermentation of the insoluble fraction were observed in dussa (DSS) and maize offal (MZO), possibly influenced by the carbohydrate fraction readily available to the microbial population (Chumpuwadee et al., 2007). Deaville and Given (2001) reported that kinetics of gas production could be affected by carbohydrate fraction.

Table 3. Gas volume and *in vitro* gas production characteristics.

Parameters	BPS	DSS	GNS	MZO	+SEM
Gas Production characteristics					
b (mL)	34.33[b]	41.33[a]	34.33d	37.00[b]	0.17
C (h^{-1})	0.0124[b]	0.0170[a]	0.0007	0.0120[b]	0.00
Gas Volume					
Gv 24h	27.33[a]	25.00[a]	18.00[b]	31.33[a]	1.16
Gv 48h	31.60[b]	33.00[a]	24.33[b]	38.67[a]	1.45
Gv 72h	34.33[ab]	37.67[ab]	30.00[b]	41.47[a]	1.65
Gv 92h	44.33[ab]	47.67[ab]	40.00[b]	51.67[a]	1.66

a,b, means on the same column with different superscripts are significantly varied (P < 0.05), b= fermentation of the insoluble but degradable fraction, c= gas production rate constant, SEM= standard error of the mean, GV = gas volume, BPS = cowpea pod, DSS =dussa, GNS= groundnut shells, MZO= maize offal, SEM= standard error of the mean.

Table 4. Metabolisable energy (ME) (MJ/kg DM), short chain fatty acid (SCFA) and organic matter digestibility (OMD).

Parameters	BPS	DSS	GNS	MZO	±SEM
ME (MJ/Kg DM)	6.69[a]	5.79[b]	3.14[c]	6.68[a]	0.06
SCFA (µM)	0.695[a]	0.687[a]	0.370[c]	0.537[b]	0.01
OMD (%)	49.63[a]	43.17[c]	39.27[d]	48.75[b]	0.07
CH_4 (mL	10.00[c]	17.00[a]	8.00[d]	13.00[b]	0.33

a,b,c, means on the same column with different superscripts are significantly varied (P < 0.05), ME = metabolisable energy, SEM= standard error of the mean, SCFA= short chain fatty acid, OMD= organic matter digestibility,CH4 = methane, BPS = cowpea pod, DSS =dussa, GNS= groundnut shells, MZO= maize offal.

The fast rate of gas produced (c) observed in dussa (DSS), beans pod (BPS) and maize offal (MZO) was probably influenced by the soluble carbohydrate fractions readily available to the microbial population. The relatively low content of fibre can facilitate the colonisation of the feed by the microbial rumen population, which in turn might induce higher fermentation rates, therefore improving digestibility (Van Soest, 1994). As the fermentation process is partially regulated by the fibrous content of the feeds, dussa ferments faster than groundnut shell (GNS), beans pod (BPS) and maize offal (MZO). Since gas production on incubation of feed is buffered rumen fluid is associated with feed fermentation and carbohydrate fraction (Sallam et al., 2008), so the higher gas production in maize offal (MZO) and dussa (DSS) could be related to fibre fraction content. This is in agreement with De Boever et al. (2005), who reported that gas production was negatively related with NDF content and positively with starch. The negative effect of cell wall content on gas production in groundnut shell could be due to reduction in the microbial activity through increasing the adverse environmental condition as incubation time progresses.

The reduction in gas and methane in groundnut shell (GNS) could be due to the conversion of CO_2 and H_2 to acetate instead of CH_4 (Miller, 1995). This is consistent with the findings of Sallam et al., (2008). The estimated ME in this study were consistent with those obtained for the concentrate feedstuff. (Chumpuwadee et al., 2007) and lower than those obtained for the different parts of *Enterelobium cyclocarpum* (Babayemi, 2006). There was a positive correlation between ME calculated and from the in vitro gas production together with CP and fat content with metabolisable energy value of conventional feed measured in vivo (Menke and Steingass, 1988). The *in vitro* gas production method has been widely used to evaluate the energy value of several classes of feed (Getachew et al., 1998, 2002). The lowest SCFA predicted from gas production in groundnut shell (GNS), due to the lowest gas production, which

was most evident during the first 24h of incubation. This is consistent with the findings of Blummel et al. (1990) who stated that different classes of feed incubated *in vitro* in buffered rumen fluid was closely related to the production of SCFA which was based on carbohydrate fermentation but did not support observation on the GNS sample in this study. A high value of SCFA is an indication of energy availability to the animal. High digestibility of organic matter (OMD) obtained in beans pods (BPS) and maize offal (MZO) is because the major carbohydrate of their feedstuffs is starch, which is fermented by amylolytic bacteria and protozoa (Kotarski et al., 1992). This result implies that the microbes in the rumen and animal have high nutrient uptake.

Conclusion

The tropical agricultural waste and by-products showed a great variation in chemical composition and mineral content. The result of this study demonstrates that gas production characteristics of the feedstuff under study differed. Based on this study, High fermentation potentials of the different agricultural wastes and by-product ranked from the highest to the lowest were: dussa, beans pod, maize offal and groundnut shells respectively.

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ANIMAL SCIENCE

Effect of arsenic exposure on metabolic activities involving adaptive responses in liver of fresh water fishes (*Channa punctata*)

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Abstract

As a major organ, liver plays the critical role in metabolic regulation, however, the metabolic activities are impaired by both environmental and chemical stimuli. *Channa punctata* variety of fishes was exposed to different concentration of sodium arsenate (Na_2HAsO_4) as the fishes are energetic and survive in the critical environment. Both 1 mM and 10 mM Na_2HAsO_4 enhanced protein content in liver for 1 h and 2 h, however, the effect was higher for 10 mM concentration. The fishes exposed to 100 mM Na_2HAsO_4 also causes increase in protein and the effect was lower than the previous doses. The inorganic phosphate (Pi) level was increased significantly whenever the fishes were exposed to Na_2HAsO_4 and found to be higher for 1 mM dose. The increased Pi release was also observed in response to higher dose, however, lower than the previous doses. Similar stimulatory effects on alkaline phosphatase activity were observed in liver exposed to the above concentrations and were found to be maximal for 1 mM dose. The results appear to indicate that the adaptive response involving the higher synthesis of molecules induced by arsenic is responsible for survive of the species and will give a new insight for the regulation of metabolic activities in liver and might be an index for characterization of the pathology of fishes.

Key words: Arsenic exposure, *Channa*, Liver, Metabolic regulation, Adaptive response

Introduction

Arsenic is an important and ubiquitous environmental toxicant and the risk of arsenic poisoning in human is a public health issue worldwide (Ahmad et al., 1997; Tchounwou et al., 2003). In addition, arsenic is classified as a human carcinogen based on several epidemiological studies showing an association of arsenic exposure with cancers in lung, bladder, kidney and liver (Hughes, 2002). Despite these findings and the fact that arsenic is the most extensively studied of the meals and metalloids in drinking water, the molecular mechanisms of arsenic toxicity is poorly understood.

The liver is a major target organ of arsenic toxicity in both mice (Waalkes et al., 2003; Wang et al., 2002) and human (Chen et al., 1986; Chen et al., 1992). Arsenic-induced liver injury in humans is a common phenomenon, typically manifesting initially as degenerative lesions with jaundice,

progressing to noncirrhotic portal hypertension, fibrosis, cirrhosis and neoplasia such as hepacellular carcinoma (Centeno et al., 2002; Lu et al., 2001). Although many arsenic-induced differentially expressed genes have been identified, there are no data on *in vivo* kinetics on the adaptive response of a specific targeted organ, such as liver, the experimental results should aid further understanding of arsenic mechanisms of toxicity resulting in pathology.

Channa punctatus is generally found in fresh water of haor, bil, river in Bangladesh. They are much energetic and survive in the critical circumstances for long time, for example, water deprivation. They are used as a source of protein in the diet for human being. It is assumed that the higher energy content of this fish is caused by the increased activity of the sympathetic nerves. During environmental adverse condition, liver might be involved critically on its regulation of metabolites to survive in the atmosphere. However, to survive in the atmosphere caused by toxic arsenic, the critical role of liver of these species on adaptive response involving the regulation of metabolic processes is not understood.

As a metabolic organ, liver plays a major role in biotransformation of foreign toxic substances. Therefore, the organ may also serve as a regulatory

area to the sensitivity of toxic substances. Liver glycogenolysis is a metabolic process by which the energy is released for doing mechanical work and there by the species may use the energy to survive. Besides glycogenolysis, other metabolic functions are also involved in the survival process, although the mechanism of the survival process and the toxic effect of arsenic causing metabolic alterations through expression of pathological syndromes are not known. Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou et al., 2003) and causes different types of pathogenic syndromes in rodents, fishes and other organisms. Exposure of higher concentration of arsenic in water may also cause severe effects in fish and might be involved in producing cancer or other cellular effects. However, the mechanism underlying the effects of acute arsenic exposure on the regulation of oxidative and glycolytic processes in liver is not well understood. The accumulation of arsenic to the living organism is mediated directly or from other species. Moreover, fish have long been used as sentinels for biomonitoring of aquatic environmental pollutants and are good indicators of arsenic toxicity (Tisler et al., 2002). The regulation of metabolic activities in liver in response to the toxic arsenic is an important aspect in fish and to clarify the role of arsenic in liver metabolic functions responsible for survive of the species of fishes in the environment and characterization of pathogenesis of this species since protein, Pi and alkaline phosphatase are the metabolic index for characterization of liver pathogenesis, the current protocol was designed.

Materials and Methods
Fishes

Channa punctatus weighing 50 g to 60 g were used. They were maintained in normal water with ambient temperature ($25.0 \pm 1.0°C$). In the day of experiment, arsenic exposure was given to the different groups of fishes in plastic pot. After the treatment, fishes were quickly decapitated and liver was sampled carefully and taken weight by digital balance (Chyo, JL-180, China) and kept at $–20°C$. Control fishes were similarly used for sampling of tissues except giving arsenic exposure.

Arsenic treatment

To examine the role of arsenic on the regulation of metabolic activities in liver, groups of fishes were exposed with different concentrations of arsenic compound (1 mM, 10 mM and 100 mM Na_2HAsO_4. $7H_2O$, BDH Chemical Ltd.). Five fishes in each group were kept in a transparent plastic pot (size 10"X10"X5"). The fishes were bought from the nearby market and maintained in normal water in the laboratory for a while without any food. During the experiment, no foods were given in the pot and both 1 mM and 10 mM arsenic exposure were done for 1 h and 2 h, however, the other groups of fishes were treated with only 100 mM of arsenic compound (Na_2HAsO_4) for 1 h in ambient temperature. The control fishes in separate pot were used with normal water only.

Assay of tissue metabolite

Liver was homogenized with pre-cooled water and was centrifuged at 8000 rpm for 10 min. The supernatant was used as crude extract for assay of protein, inorganic phosphate and alkaline phosphatase (ALKP) activity. Protein was determined by Lowry et al. (1951) method by using 50 μl crude extract. Inorganic phosphate and alkaline phosphatase activity were determined as described by Ramnik (1999) by using 100~200 μl extract. For Pi estimation, 200 μL tissue extract was diluted to 5 mL with water and was mixed vigorously with 5 mL of 5% TCA (Trichloroacetic acid) and centrifuged at 6000 rpm for 10 min. 5 mL supernatant was transferred to another tube and kept on ice. 1 mL molybdate reagent (10 g of ammonium molybdate in 100 mL water was taken and 100 mL of 5N H_2SO_4 was added to prepare 200 mL solutions) was added and mixed. The solution was mixed with 0.4 mL aminonaptholsulphonic acid reagent. 3.6 mL water was added and after mixing, the tube was kept standing for 10 min for the complete development of color. For blank, 5 mL of 5% TCA and 5 mL water were mixed only. Absorbance was taken at 690 nm against the blank. The Pi in tissue extract was calculated using standard KH_2PO_4 solution.

For assay of alkaline phosphatase activity, 0.25 mL of PNPP (p-nitrophenyl phosphate (1.2 mg/mL in glycine-NaOH buffer, pH 10.0) was added to 0.5 mL glycine-NaOH buffer (pH 10.0) and incubated for 5 min at 37°C. 100~200 μL of tissue extract was taken to the solution and for blank, same volume of buffer was used in place of tissue extract and incubated for 30 min. After incubation, it was made up to 4 mL with 0.1N NaOH solution and absorbance was taken at 410 nm. The amount of PNP (p-nitrophenol) produced after hydrolysis of PNPP) by the enzyme was measured from the standard PNP solution (500 $μmolL^{-1}$ in buffer, pH 10.0). The enzyme activity is expressed as μmol of PNP $min^{-1}g^{-1}$ of tissue.
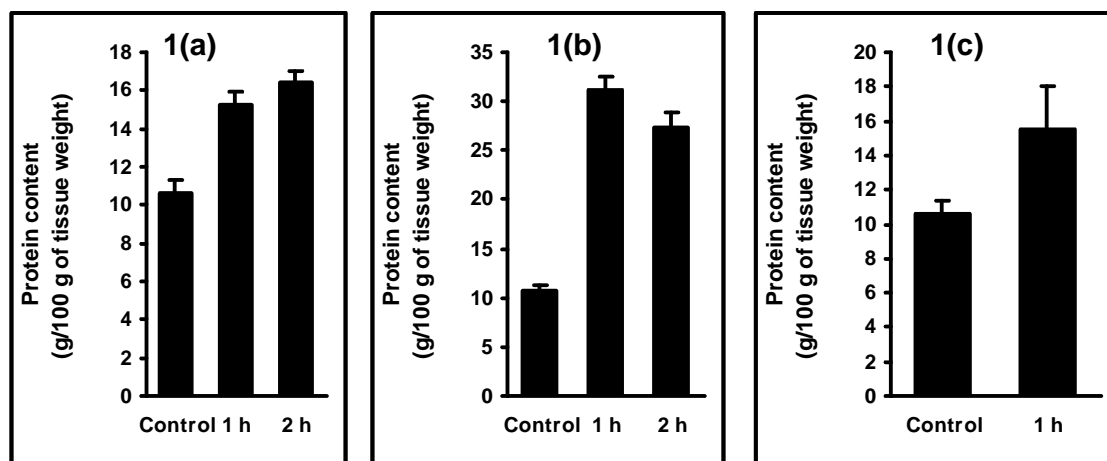
Figure 1. Changes of protein content in liver of fishes exposed to 1 mM (a), 10 mM (b) and 100 mM (c) Na_2HAsO_4. The fishes were kept for 1 h and 2 h in arsenic solution, however, for 100 mM concentration; they were kept for 1 h. The data are average $\pm$ SEM for 4~5 fishes in each group.

Statistical analysis

Results of the experiments were expressed as mean and standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by pared t-test using SPSS software.

Results

Effects of 1 mM, 10 mM and 100 mM Na_2HAsO_4 on protein content in liver

As shown in Figure 1 (a), the average protein in liver in response to 1 mM arsenic for 1 h and 2 h were 15.24 ± 0.73 g and 16.47 ± 0.53 g respectively whereas for control fishes, the amount of protein was 10.63 ± 0.72 g/100 g of tissue weight. A significant 43.36% ($P<0.001$) and 54.93% ($P<0.05$) increased protein was observed after 1 h and 2 h respectively when compared with control liver. Groups of fishes were exposed to 10 mM arsenic solution and the amounts of protein for 1 h and 2 h were 31.20 ± 1.22 g and 27.37 ± 1.53 g/ 100 g of liver respectively. The results indicated that 193.50% ($P<0.001$) and 157.47% ($P<0.001$) increased protein had been found respectively after 1 h and 2 h in response to 10 mM Na_2HAsO_4 compared to the control fishes, shown in Figure 1 (b). The results also appear to indicate that 10 mM arsenic causes higher protein content than 1 mM concentration.

The amount of protein in liver of other groups of fishes in response to higher arsenic level (100 mM Na_2HAsO_4) for 1 h was 15.57 ± 2.42 g and for control, the value was 10.63 ± 0.72 g /100 g of tissue weight, shown in Figure 1 (c). The results

indicate that 100 mM Na_2HAsO_4 causes also increased protein by 46.47% ($P<0.1$), however, lower than the previous doses.

Effects of 1 mM, 10 mM and 100 mM Na_2HAsO_4 on inorganic phosphate (Pi) level in liver

To examine the role of arsenic on Pi release, fishes were exposed to 1 mM Na_2HAsO_4 for 1 h and 2 h and the amount of Pi releases were 12.67 ± 0.79 mg and 12.76 ± 1.33 mg /100 g of tissue weight while for the control fishes, the value was 2.68 ± 0.19 mg/100 g of tissue. The Pi values in livers of arsenic-treated fishes were increased significantly by 372.76% ($P<0.001$) and 376.11% ($P<0.001$) respectively for 1 h and 2 h than in livers of control fishes, Figure 2 (a). In separate examinations, groups of fishes exposed with 10 mM Na_2HAsO_4 had 9.26 ± 0.78 mg and 3.42 ± 0.33 mg Pi after 1 h and 2 h respectively. The Pi content in liver was increased similarly significantly by 245.52% ($P<0.001$) and 27.61% ($P<0.05$) respectively when compared to the control as demonstrated by Figure 2 (b). The results demonstrated that arsenic causes stimulatory effect on Pi release, however, the effects were more pronounced when the fishes were exposed to 1 mM arsenic compound than 10 mM concentration.

As shown in Figure 2 (c), the increased Pi content in liver in response to 100 mM Na_2HAsO_4 for 1 h was 3.59 ± 0.59 mg while for control fish, the value was 2.68 ± 0.19 mg/100g of tissue. Arsenic causes increased Pi by 33.95% ($P<0.001$) and the effect was lower than the previous doses.
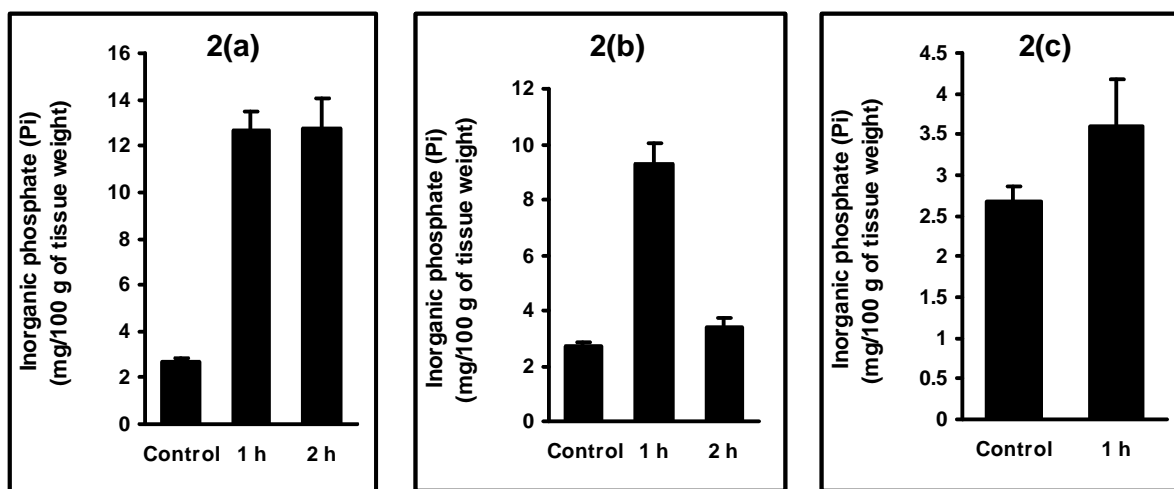
Figure 2   Changes of inorganic phosphate (Pi) in liver of fishes exposed to 1 mM (a), 10 mM (b) and 100 mM (c) Na_2HAsO_4. The fishes were exposed to arsenic for 1 h and 2 h, while for 100 mM concentration; they were kept for 1 h. The data are average $\pm$ SEM for 4~5 fishes in each group, however, 4 fishes were used for 100 mM concentration only.

Therefore, it is assumed that the lower arsenic level might be effective for producing higher Pi release from the liver. The results suggest that the increased Pi might be due to the higher activity of some enzymes responsible for the degradation of the cellular organic compounds and could be considered as the survival factor for this species in critical environment.

Effects of 1 mM, 10 mM and 100 mM Na_2HAsO_4 on alkaline phosphatase (ALKP) activity in liver

Liver ALKP is sensitive to toxic response and releases Pi from PNPP. To examine the role of arsenic on ALKP activity in liver, groups of fishes were treated with 1 mM and 10 mM Na_2HAsO_4 for 1 h and 2 h and the control livers of fishes kept in ambient temperature were also examined. The ALKP activities in response to 1 mM Na_2HAsO_4 for 1 h and 2 h were 2.68 $\pm$ 0.21 μmol and 2.70 $\pm$ 0.23 μmol respectively whereas for control fishes, the activity was 0.35 $\pm$ 0.08 μmol/min/g of tissue. The results demonstrated that ALKP activities had been significantly enhanced and stimulated (665.71%, $P<0.001$ and 671.42%, $P<0.001$ respectively) by arsenic compared to control, however, the activity was appeared to be higher for 2 h exposure as shown in Figure 3 (a). The ALKP activities in livers of other groups of fishes in response to 10 mM Na_2HAsO_4 were 1.28 $\pm$ 0.04 μmol and 1.00 $\pm$ 0.05 μmol respectively. A significant increased response on ALKP activity was observed for fishes exposed to arsenic (265.71%, $P<0.05$ for 1 h and 185.71%, $P<0.01$ for 2 h), Figure 3 (b), however, the effects were lower than 1 mM concentration, Figure 3 (a), Figure 3 (b).

As shown in Figure 3 (c), the ALKP activity in presence of 100 mM Na_2HAsO_4 for 1 h was 0.93 $\pm$ 0.12 μmol while for control fish, the value was 0.35 $\pm$ 0.08 μmol/min/g of tissue. Arsenic causes increased activity ($P<0.05$) by 165.71%, however, the effect was lower than the previous doses. The results suggest that the increased ALKP in liver might be due to the toxic effect of arsenic which produces the toxic environment where they survive and could be considered as the survival factor as well as index for characterization of the pathology of liver for this species.

Discussion

The increased protein caused by different concentrations of arsenic (1, 10 and 100 mM Na_2HAsO_4) exposure has been found in liver of fishes in our study. Arsenic produced the toxic environment in water where the fishes want to survive. Therefore, it is reasonable that an adaptive response by the species was created and some stress proteins were synthesized. The liver is the organ where most of the biotransformation of inorganic arsenic takes place (Del Razo et al., 2001). Up regulation of several genes in arsenic-induced adaptive response has been observed (Verma et al., 2002; Chelbi-alix et al., 2003). Their findings suggest that arsenic may induce the synthesis of molecules responsible for the survival process. The liver is a major target organ of arsenic toxicity.
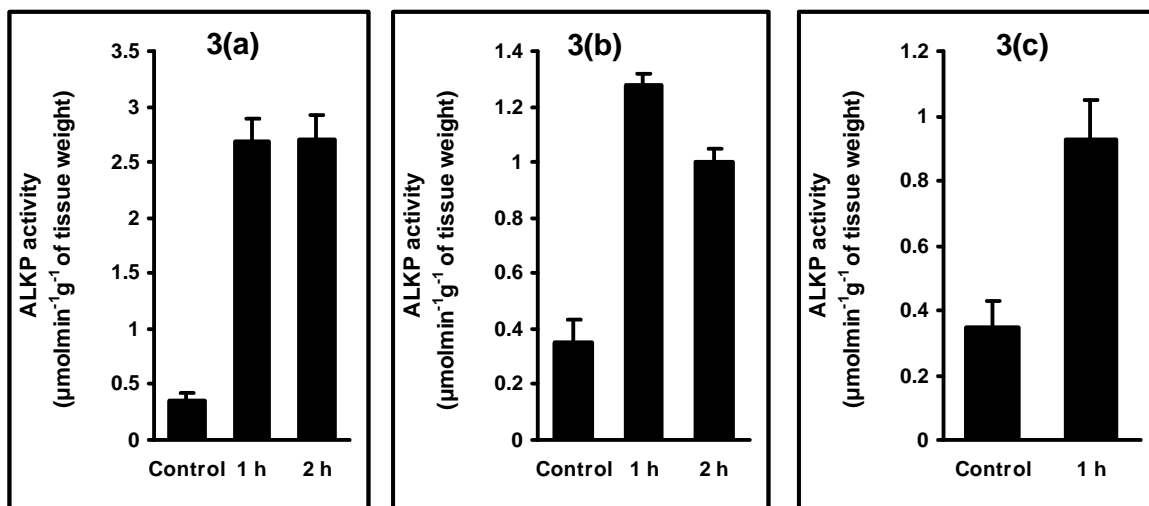
Figure 3 Changes of alkaline phosphatase (ALKP) activity in liver of fishes exposed to 1 mM (a), 10 mM (b) and 100 mM (c) Na_2HAsO_4. The groups of fishes were exposed to arsenic solution for 1 h and 2 h, while for 100 mM concentration; they were kept for 1 h. The data are average ± SEM for 4~5 fishes in each group, however, 4 fishes were used for 100 mM concentration only.

Arsenic-induced liver injury in humans is a common phenomenon, typically manifesting initially as degenerative lesions with jaundice, progressing to noncirrhotic portal hypertension, fibrosis, cirrhosis, and neoplasia such as hepatocellular carcinoma (Mazumder et al., 1998; Lu et al., 2001; Centeno et al., 2002). Prolonged exposure of higher concentration of arsenic has been involved in liver injury and damage (Taylor et al., 1989; Chiou et al., 1995). Liver metabolism is a known potential target for the toxic action of chemicals (Hinton et al., 2001).

Severe Pi release was observed in response to 1 mM Na_2HasO_4, however, both 10 mM and 100 mM Na_2HasO_4 also induced Pi release significantly in liver. It is assumed that 1 mM arsenic effectively and maximally enhanced Pi content. Several molecules might be involved for causing Pi release. The phosphorylase enzymes and phosphatases release Pi whenever they are activated. In the biological system, phosphorylase and the phosphatase are dominantly expressed and are involved in releasing inorganic phosphate. Because of the importance of Pi in biological systems and in medical analysis, a variety of assays have been developed for this ion. Even ALKP also causes the release of Pi upon activation (Nixon et al., 1998; Christenson, 1997). We found that ALKP was significantly increased in response to different concentration of arsenic. Further experiments are needed for clarification of the mechanism of enhancing the Pi release in liver of fishes treated with arsenic. Allen et al. (2004) found that arsenic impairs the sympathetic nerve activity; therefore, the toxic arsenic might be involved in denervation of the similar nerves. Therefore, sympathetic denervations induced by arsenic may play the role for changing the amount of Pi through phosphorylation-dephosphorylation reactions since the tissue is enriched with the similar nerves. It has been demonstrated that inactivation of the protein PP2A is mediated by cold acclimation recognized to be the major sympathetic stimulus (Leduc, 1961) resulting enhancement of phosphorylation process (Antonio et al., 1998). Therefore, the increased Pi in response to arsenic in liver might be due to the higher dephosphorylation process. Because Ca^{2+} enhances the inactivation of protein phosphatase 2A (PP2A) (Antonio et al., 1998), it could be a negative modulator for the higher Pi release. As a peripheral tissue, liver may participate in the survival process during critical circumstances through protein phosphorylation-dephosphorylation mechanisms.

Alkaline phosphatase is predominantly found in liver and is an index of the characterization for liver pathogenesis. The major isozymes of ALKP include those of liver, bone and kidney and those of the intestine and the placenta; the isozymes of these two groups are each encoded by a separate gene (Toe et al., 1989; Weiss et al., 1989). Although this glycoprotein is widely distributed in vertebrate

tissues, its physiological function is as yet fully understood. The released Pi might be influenced in response to low dose of arsenic (1 mM) because ALKP activity is severely augmented than other doses, however, the activity was appeared to be reduced in higher concentrations. The results would indicate that metabolic function involving higher ALKP activity in liver is sensitive to this dose. Recent investigation revealed that arsenic exposure stimulated ALKP in liver (Sharma et al., 2007) and is there by supported the present findings. The higher effect of arsenic in liver demonstrates that increased Pi release would be a survival factor during energy deficiency.

Collectively, as a peripheral tissue, liver is metabolically important for energy consumption and energy expenditure. Environmental toxic arsenic is a major stimulus exerting its effect on metabolic changes. As a major metabolic site, liver plays the critical role in the biotransformation of foreign toxic substances. The diverse metabolite regulation is an index concerning survival of these species as well as characterization of liver pathogenesis and is a biological process; however, arsenic probably takes part in modulation of the metabolic process.

Acknowledgement

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AGRICULTURAL ECONOMICS

Agro-ecological evaluation of tropical farming systems using emergy: in Rio de Janeiro – Brazil

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Abstract

The article is based on an agro-ecological evaluation of seven different agricultural systems in Teresópolis, Rio de Janeiro. The studied systems are dealing with: vegetable production system, ecological husbandry, cattle production system, sylvopastoral approaches as well as citrus cropping systems. The main objective is to evaluate the environmental impact of these systems using the "Emergy Analysis" as methodology. For this purpose, input data as materials, services, natural renewable/nonrenewable sources of 42 crops were analyzed. This method is based on energy flows, transforming all inputs and outputs in a common unit. This analysis allows comparisons across agricultural systems and their environmental impacts, as well as, makes possible the identification of scenarios to achieve greater sustainability. The main conclusions of this study are: the vegetable systems have large amounts of energy invested in irrigation, fertilizers and fuels; the largest value of sustainability corresponds to the ecological systems and it has the capacity to save capital in form of biomass in the system; cattle system causes bigger environmental damage and have the smallest yield per hectare in economic and energy terms; as for the citrus systems, a low investment rate was found and the use of renewable resources from this system is comparable to the vegetable systems.

Key words: Emergy analysis, Farming systems, Agro-ecology, Atlantic Forest

Introduction

This study was carried out in the basin of Córrego Sujo, in the municipality of Teresópolis, located in the Atlantic rainforest, in the hinterland of Rio de Janeiro. The basin is composed for 9 micro-basins, covering an area of 53 km^2 (Homma, 2003; Gaese et al., 2009). The agriculture in the region is characterized by intensive, small (less than one ha) but often irrigated horticultural production systems (Gaese et al., 2005). This horticultural system has little or none interaction with the cattle and forest subsystem. The inputs such as organic and inorganic fertilizers are introduced to the system (Torrico, 2009). The plants are produced in the region using good quality seed. Most of the young plantlets are produced locally in specialized nurseries. The products of the

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system are marketed by different channels, mostly dominated by middlemen who take the production to the surrounding markets. The productive units generally opt for diversification market strategies, since the prices are quite fluctuating during the whole year (Blumen, 2006).

The horticultural systems in the region are highly intensive, especially the horticultural systems based on either fruit, leaf or mixed that make high use of inputs like nitrogen fertilizers, pesticides and herbicides. These systems are also the most common ones in the region. The ecological system hardly makes use of external inputs. The cattle system occupies notably the biggest territorial extension, 83.7% of the total agricultural area (Meier et al., 2006) and it uses low external inputs as is also the case for the sylvopastoral and citrus systems (Torrico, 2004a).

The objective of this paper is to evaluate the environmental impact of the seven farming systems, the load capacity and the use of natural and economic resources using a common unit, based on the solar energy reaching the earth. The hypothesis is: quantifying inputs of agricultural systems on a common basis using emergy analysis, will facilitate comparisons across agricultural systems and its environmental impacts, as well as,

make possible the identification of scenarios to achieve greater sustainability (Odum, 1988, 1996). It is assumed that agricultural systems can be compared not only with each other but also with the surrounding natural systems, using the same units. This comparison is possible through the emergy theory that is a powerful method to measure the environmental and economic impacts caused by agricultural systems. This hypothesis also refers to the loss of natural capital of a region, and to the quality of the inputs. It allows identifying different scenarios, within which it is possible to replace less efficient systems through others with the objective to increase sustainability and to balance natural and agricultural systems.

Materials and Methods

This study was carried out in the Córrego Sujo Basin in Teresópolis. In the municipality of Teresópolis in the mountain region of the Atlantic Forest, emergy analysis was accomplished to compare the main Farming systems in the Córrego Sujo basin. To evaluate the environmental impact, the input data and average yields of 42 crops. To accomplish this objective we have analyzed: (a) ecological (bio) farm, (b) cattle systems, (c) fruit vegetables, (d) leaf vegetables, (e) vegetables mixed systems and (f) citrus plantation. As materials we consider: seeds, limestone, fertilizers, pesticides, herbicide, fuels, and machinery (the latter one considered as depreciation of capital investment); as services we consider: manpower, administration, transport, taxes, insurance, and social security. The manpower data have been expressed in terms of working days of 8 hours $ha^{-1}year^{-1}$ i.e. $md\ ha^{-1}y^{-1}$.

For this purpose, classical economic analysis and emergy analysis was required in order to compare the main farming systems of the basin. The used economical indices were internal rate of return, cash flow, net present value and investment cash flows.

The emergy methodology is a quantitative evaluation method which valorises the nature input to the economic systems. In other words, emergy is a measure of direct and indirect supporting energy needed in different work processes supporting a product or a service (money, mass, energy, information), using a common unit (Odum, 1996; Brown, 2001). In this method, the basic unit of measurement used is the solar emergy joule (sej) (sej = solar energy expressed in Joul), which refers to the accumulated amount of energy used up in the chain behind a good or service (Odum, 1988, Odum and Odum, 2000. The solar transformity of sunlight

absorbed by the earth is defined as the baseline at 1 sej J^{-1} Corresponding to a yearly input of 3.65E+13 J/ha. The basic idea is that solar energy is our ultimate energy source and by expressing the value of products in emergy units, it becomes possible to compare apples and pears (Jorgensen, 2001).

The procedure for the emergy evaluation is described and summarized by Haden (2003) in three steeps: the first one consists of drawing the energy system diagram, the second one elaborates the emergy evaluation table and the third one the calculation of the emergy indicators as well as the summary diagrams. The summary diagrams shows all aggregated emergy inputs coming from the economy system as services or materials and from the natural system as renewable or not renewable resources. In Figure 1, R is the sum of the renewable emergy flows supporting the economy (i.e. rain, waves, tide); N is the sum of non-renewable resources from within the system (national) boundary; M is the sum of all materials used or paid in the system; S is the sum of all services used or paid in the system; Y is the total consumed emergy; Ep is the total energy produced from the system and C is the capital of the system (biomass, biodiversity, water, soil fertility, etc).

After tabulating the material and energy flow data for the system in question and correcting for their emergy contributions using transformities[1] (Odum, 1988; Scienceman, 1987, 1989), a number of emergy ratios and indices were calculated (Table 1). For all these calculations emergy related indices have been introduced to assess various aspects of the sustainability for farming systems. The transformity is the inverse value of the system efficiency for a product or service). In 1996 H. T. Odum defined transformity as, "the emergy of one type required to make a unit of energy of another type. For example, since 3 coal emjoules (sej) of coal and 1 sej of services are required to generate 1 J of electricity, the coal transformity of electricity is 4 sej J^{-1}". The Emergy Yield Ratio evaluates the efficiency of a production unit or process. If the relationship is smaller than 1 the system consumes more than what it produces (Ortega, 2001). The Environmental Load Ratio measures the environmental impact, especially through soil loss (Chen, 2003; Lal, 2003). When the relation has a

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1 In Scienceman (1987) proposed that the phrases, "energy quality", "energy quality factor", and "energy transformation ratio", all used by Odum, be replaced by the word "transformity" (p. 261). This approach aims to solve a long standing issue about the relation of qualitative phenomena to quantitative phenomena often analysed in the physical sciences, which in turn is a synthesis of rationalism with phenomenology. That is to say that it aims to quantify quality.

high value it suggests that the system uses high technological levels in terms of emergy. The Emergy Investment Ratio measures the dependence of the system from bought products, and indirectly measures the environmental loads. The value increases proportionally with the dependence. The Emergy Exchange Ratio measures the capital loss

of the system (Cavaletta, 2004). If the values are smaller than 1, it means that the system transfers positively to the economic urban system. The Renewability indicates the percentage of renewable emergy in relation to the total emergy used from the system.
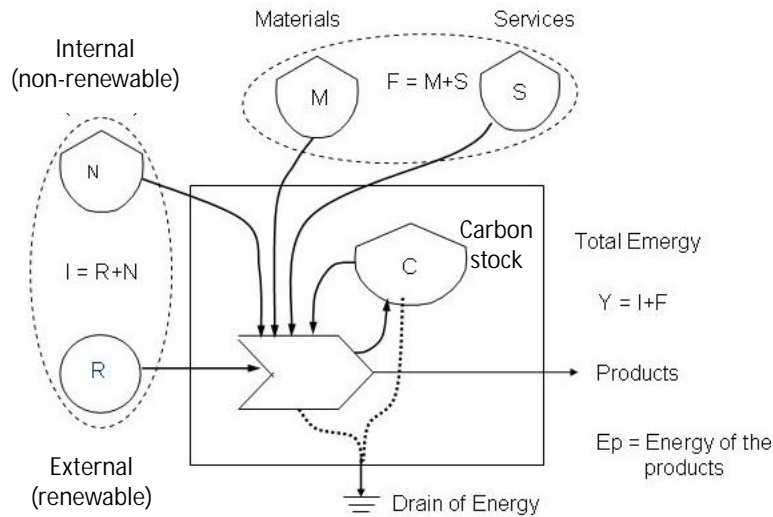

Figure 1. Aggregated emergy input and outputs from the economy (service and materials) and renewable and not renewable resources from natural systems.

Table 1. Summary of the emergy indices used in this study.

Indices	Form	Description
Emergy Yield Ratio	EYR = Y/F	Evaluates the efficiency of a production unit or process. If the relationship is smaller than 1 the system consumes more than it produces;
Environmental Load Ratio	ELR = (N+F)/R.	A measure of environmental impact. A high value indicates heavy dependency on non renewable energy sources.
Emergetic Investment Ratio	EIR = F/I.	Measures the dependence of the system on purchases material and services, and indirectly measures the environmental loads;
Emergy Exchange Ratio	EER= Y/income*3.18E12	Measures the capital loss of the system. If the value is lower than 1, it means that the system transfers positively to the urban economy;
Transformity	Tr = Y/Ep (sej/J)	Is the amount of energy (expressed in sej/J or sej/g), which has been used to create a flow or resource;
Renewability	%R = R/Y*100 (%)	Indicates the percentage of renewable emergy in relation to the total emergy used from the system.

Source: Adapted from Odum (1996)

sej = Solar energy expressed in Joul

3.18E12 = setting value from regression of Brazilian GDP

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Results and Discussions
Farming systems

All physical, biological and monetary inputs of the studied agricultural systems were converted into emergy flows and are aggregated as shown in Figure 2 drawn based on Odum (1994) and Ortega, (2001). The principal renewable flows are sunlight, rainfall and minerals. Purchased goods, fertilizers, fuels, and services are also shown. Internal production systems include forests and forest in regeneration (1 to 3 years old), citrus orchards, intensive and ecological-organic farming systems; livestock are also shown in Figure 2.

Positive economic indices were recorded for all crops cultivated as a monoculture system. The relationship costs benefit indicates that all crops recover more money than the amount invested. The cultivations with more values are: Onion Evergreen (9.87), Paprika (3.7), and Tomato (2.99). The cultivations that present smaller relations of benefit cost are: cabbage (1.12), endive (1.18) and beet (1.22) (Table 2). Because the crop cycle is always smaller than 1 year, the recovery of capital is quick.

From that point of view the Net Present Value as well as the Internal Rate of Return are highly positive (77), whereas the average net income per hectare and per year is R\$ 15,500 for average production costs amounting to R\$ 8,200 per ha and year.

The intensive horticultural systems for fruits, leaves or mixed systems are presenting big economic returns, varying according to the fluctuating market prices. The revenues can reach 3,600 up to 13,480 US\$ ha^{-1}. On average, revenues for the horticultural systems based on fruit, on leaf or on mixed production amount to 6,760, 4,770 and 5,110 US\$ ha^{-1} respectively. From the economic point of view, large differences exist with the less intensive systems, like the ecological systems with 2 to 6 months fallow and having a net annual income of 899 US\$ ha^{-1} on average. Finally, the systems that present very low income per hectare and per year are the Cattle, Sylvopastoral and Citrus systems with 78, 84, and 146 US\$ ha^{-1}y^{-1}, respectively (Table 3).
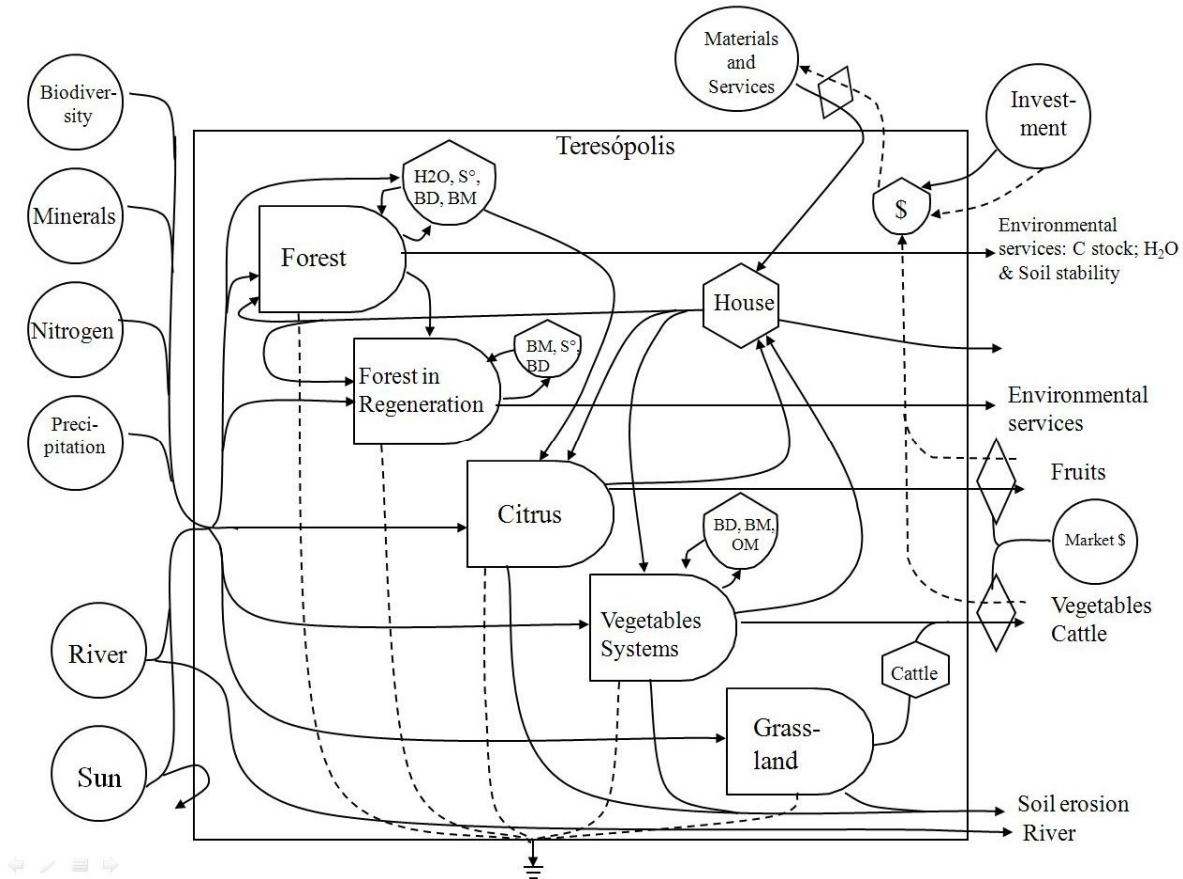

Figure 2. Teresopolitan environmental/economic system depicting resource flows entering the system and the organization of major internal components that utilize those resources. BD: Biodiversity, BM: Biomass, OM: Organic Matter and S: Soil.
(Diagram based on Odum 1994 and Ortega 2001).

Table 2. Calculation of economic indicators of the most important crops (as monoculture).

Crop	Production Cost (R$[3] ha^{-1})	Gross income (R$ ha^{-1} y^{-1})	Indicators IRR[1]	C/B[2]
Lettuce	4,869	8,478	35	1.74
Water cress	6,201	16,744	54	2.70
Broccoli	10,255	20,513	15	2.00
Onion Evergreen	12,350	121,900	71	9.87
Chicory	7,061	19,110	83	2.71
Lady finger	12,652	23,360	29	1.85
Chinacol	14,833	35,633	47	2.40
Cilantro	6,236	19,295	164	3.09
Spinach	6,883	18,272	135	2.65
Paprika	10,826	40,016	74	3.70
Tomato	14,004	41,939	111	2.99
Carrot	2,433	5,760	28	2.37
Zucchini	3,758	7,336	38	1.95
Endive	5,743	6,750	33	1.18
Beet	5,223	6,380	41	1.22
Col	5,427	15,170	102	2.80
Mint	8,435	2,620	88	1.50
Leek	12,067	42,140	79	3.49
Rocket	5,728	15,189	54	2.65
Cabbage	13,200	14,750	62	1.12
Rocket	5,728	9,600	65	1.68

(1) Intern Rate of Return,

(2) Cost/Benefit,

(3) 1.00 BRL = 0.583245 USD FEB 21, 2012)

Table 3. Average Net Income of the most important farming systems in Córrejo Sujo

Farming system	Range of Net Income (US$ ha^{-1}y^{-1})	Average Income (US$ ha^{-1}y^{-1})
Bio-farm	120 to 2,450	899
Cattle	66 to 98	78
Sylvopastoril	66 to 102	84
Fruit Vegetables	4,440 to 10,220	6,760
Leaf Vegetables	3,600 to 12,780	4,770
Mixed Vegetables	4,800 to 13,480	5,110
Citrus	130 to 189	146

Source: author based on Torrico (2004a and 2004b)

Manpower

The agricultural systems that use more manpower are those based on horticulture, either for fruit, vegetable leaves or mixed production, using in total 118 workers/100 hectares, corresponding to 80% of the available household manpower. The vegetable systems that conserve a forest fragment (60% of the area) in the production unit use considerably less manpower, approximately 45 workers per 100 ha, of which 65% correspond to family manpower. The ecological systems use low quantity of manpower per production unit, since usually the areas dedicated to the agricultural production do not surpass 10% of the total area (Figure 3).

The horticultural systems of the region are highly intensive, especially the horticultural systems based on either fruit, leaf or mixed production that make high use of inputs like nitrogen fertilizers, pesticides and herbicides. These systems are also the most common ones in the region. The ecological system hardly makes use of external inputs. The cattle system occupies notably the biggest territorial extension (83.7% of the total agricultural area) and it uses low external inputs as is also the case for the sylvopastoral and citrus systems. A general overview of the farming systems in Córrego Sujo Basin is presented in the Table 4.
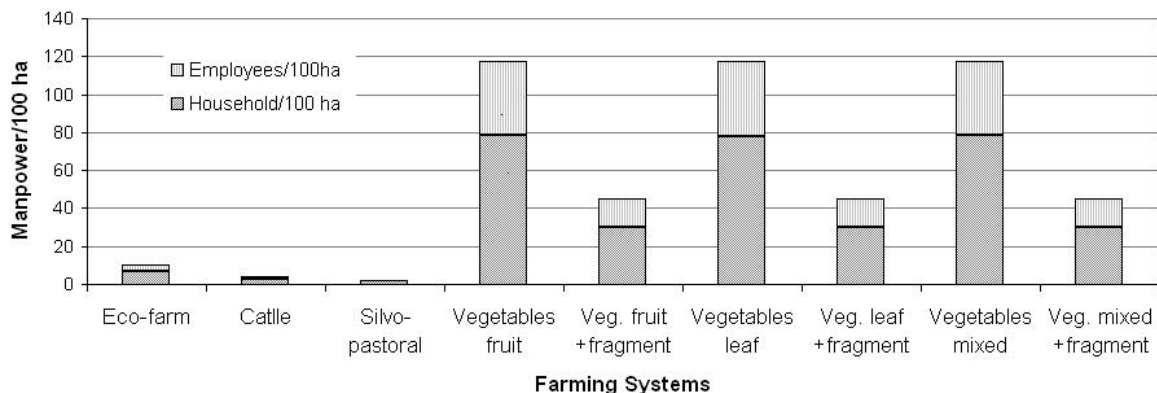
Figure 3. Family and employed manpower in different farming systems.

Table 4. General overview of the agriculture systems in Córrego Sujo, Teresópolis.

	Ecofarm	Cattle	Sylvo-pastoral	Fruit Vegetables	Leaf Vegetables	Mixed Vegetables	Citrus
Area (%)*	0.1	83.7	2.3	2.9	5.8	2.2	2.8
Seeds quality	high	none	none	high	very high	very high	high
Fertilizers	none	none	none	high	high	high	low
Pesticides	none	none	none	high	high	high	none
Herbicides	none	none	none	moderate	moderate	moderate	none
Anti-parasites	none	moderate	moderate	none	none	none	none
% Forest (average)	80	5	15	33	32	32	15
% Crops Area (average)	18	0	0	66	66	66	84
Fallow (months/yr)	2 to 6	0	0	0	0	0	0
Production Losses (%)	18	0	0	14	11	11	10
Market destination (%)	20	100	100	99	100	100	98
Irrigation	low	none	none	high	high	high	None
Principal product	diversified	meat	meat	Chayote, tomato	salad, cabbage	Chayote, salad	Mandarin
* Percent of the total agricultural area in Teresópolis							

Emergy synthesis

A farming system is a natural resource management unit operated by a farm household, and includes the entire range of economic activities of the family members (on-farm, off-farm agricultural as well as off-farm non-agricultural activities) to ensure their physical survival as well as their social and economic well-being (Lockeretz, 1982; Chen, 2006).

Emergy synthesis for each farming system is summarized in the Table 5. The long-term sustainability of human economic production and its basis in natural capital stocks is achieved via a suite of emergy-based indices (Unicamp, 2004; Haden, 2003). These indices, which relate flows from the economy to flows to the environment,

were used to compare net yields and environmental loading, and to identify more sustainable agricultural methods. The fraction Renewability (%R) quantifies the reliance of each system on renewable energies. The Emergy Yield Ratio (EYR) compares units of exported energy with emergy invested. For agriculture, an Emergetic Investment Ratio (EIR) from the economy is made in order to capture renewable emergy from the environment. This ratio quantifies the effectiveness of non-renewable resources to capture renewable resources. The Environmental Loading Ratio (ELR) is the ratio of purchased and non-renewable resources to renewable resources (Brown and Ulgiati, 1999; Brown, 2008).

Table 5. Computed transformities and emergy indices for farming systems in Córrego Sujo (Teresópolis) and comparison with literature.

Emergy indices from farming systems in Teresópolis	Indices					
	T[1]	%R[2]	EYR[3]	EIR[4]	ELR[5]	EER[6]
Ecofarm	4.8E4	66	5.34	0.23	0.51	1.23
Cattle	6.3E7	41	22.56	0.05	1.41	0.47
Sylvopastoral	2.3E5	41	19.16	0.06	1.44	0.43
Fruit vegetables	3.1E5	15	1.25	4.02	5.66	0.61
Leaf vegetables	6.7E5	12	1.19	5.26	7.28	0.92
Mixed vegetables	4.3E5	13	1.21	4.68	6.52	0.61
Citrus	3.4E5	43	2.78	0.56	1.35	1.91
Córrego Sujo (average of all farming systems)						
Emergy indices for different farming systems (literature)						
Ecological soybean [a]	8.8E4	92	1.09	1.19	0.46	1.45
Organic Soybean [a]	8.1E4	78	1.27	1.40	0.42	1.35
Chemical Soybean [a]	1.0E5	74	1.35	3.40	0.23	2.51
Herbicide Soybean [a]	1.1E5	31	3.25	3.70	0.21	2.69
Ecological farming system [b]	2.0E5	69	3.36	0.4	0.82	0.02
Ecofarm integrated system [c]	2.8E5	75	11.90	0.09	-	5.52
Sitio Santa Helena [c]	8.5E5	27	2.52	0.66	-	2.33
Sitio Três lagos [c]	2.3E6	25	7.82	0.15	-	9.91
Bovine meat (sej kg^{-1}) [d]	2.1E12*	8	7.83	-	11.0	-
Danish agriculture [e]	-	-	1.17	5.91	9.67	-

* sej kg-1

(a)Ortega (2001), (b)Unicamp (2004), (c)Roosevelt-Agostino (2001), (d)Serrano (2001), (e)Haden (2003)

(1)Transformity; (2)Renewability; (3)Emergy yield ratio; (4)Emergy investment ratio; (5)Environmental loading ratio; (6)Emergy exchange ratio

Transformity (Tr)

Transformity values per ha varies from 4.88E4 to 6.30E7 sej J^{-1}. The transformity values of ecological systems (4.88E4 sej J^{-1}) are lower than that of the systems like cattle (6.30E7 sej J^{-1}), Silvopastoral (2.35E5 sej J^{-1}), vegetables on average (4.5E5 sej J^{-1}), and citrus (3.3E5 sej J^{-1}). This means that ecological systems are more efficient, whereas cattle systems are most inefficient. Other agricultural transformities are reported by Brandt-Williams (1999) in Florida for corn (1.26E5 sej J^{-1}) and tomatoes (8.6E5 sej J^{-1}), by Cohen (2006) for maize in Kenya (1.11E5 sej J^{-1}), by Ortega et al. (2001) in Brazil for Ecological soybean (8.8E4 sej J^{-1}), for Chemical soybean (1.0E5 sej J^{-1}), and by Haden (2003) in Denmark for crops and animal husbandry (2.59E5 sej J^{-1}).

Renewability (%R)

Is the percentage of the total energy driving a process that is derived from renewable sources (%R = R/Y). In the long rung, only processes with high %R are sustainable. As renewable resources we consider: rain, uptake of nutrients like nitrogen, minerals from soil rocks, products and services obtained from the farm area under preservation (Ortega, 2001), according to Brazilian law at least 20% of total area (Brazil, 2009).

Because of the large amount of non-renewable inputs relative to renewable inputs, the vegetable system had the lowest fraction of renewable inputs (12%) compared to the citrus system (43%) and to the ecological system (66%). This indicates that the ecological system depended on renewable resources for over 66% of its inputs meaning that from an ecological point of view it is the most sustainable. Other renewability ratios for agricultural systems are presented in Table 4. The EIR, ELR and EYR offer additional information about the ability of each land use to be related to the larger economic system.

Emergy Yield Ratio (EYR)

Because the cattle and silvopastoral systems are based almost exclusively on natural inputs, the EYR ratios are as high as 22.6 and 19.2, respectively, as would be expected. This indicates that these systems incorporate high free resources from nature in to the society or economy systems, but with a high loss of non-renewable resources (erosion). The ecological system has strong internal recycling which renders economic benefit to the farmer and ecological benefit to environment. The ecological (biological) system value is 5.4. The EYR typical values for agricultural products vary from 1 to 5. The lowest value is one, which happens when nature inputs are null (RN = 0). The

difference above the minimum value measures the cost-free contribution of the environment to production.

The value of EYR for the vegetable systems is closest to unity (1.19, 1.21, 1.25); it means that the nature contribution is low when compared to resources from economy; so, this system is not able to deliver too much net emergy to consumer systems because most parts of inputs are not renewable (e.g.: herbicide, fuel, fertilizers, pesticides, etc.). For the citrus system the value is slightly higher (2.78), this system do not have high economy inputs, and natural resources are bigger. The ecological system has strong internal recycling which renders economic benefit to the farmer and ecological benefit to environment. Bastianoni and Marchettini (2001) found an EYR value of 1.96 for farms with six different crops and livestock in Italy.

Emergy Investment ratio (EIR)

The intensive vegetable systems values are high (4.02 to 5.26), thus demonstrating an economically fragile agriculture due to its dependence on purchased inputs from foreign regions. The citrus system has good value (0.56). Livestock production, sylvopastoral systems and the Ecological farm show the lowest values, 0.05, 0.06 and 0.23 respectively. Those three systems use nature resources (free) instead of economy resources (expensive) having lower need of external investment and lower production costs. The ecological option demands more economy inputs (services) than the cattle systems. More emergy investment ratios for agricultural systems are presented in Table 5.

Emergy Load Ratio (ELR)

Vegetables leaf, fruit and mixed systems (7.28, 5.66 and 6.52) produce great environmental damage. Also the cattle systems, silvopastoral systems and citrus systems (1.41, 1.44 and 1.35) generate high environmental impact. Ecological agriculture instead has lower value (0.51), which confirms greater use of natural renewable resources by ecological and organic production techniques. The greater environmental loading ratios for the intensive vegetable systems and cattle systems compared to the ecological system reflect the environmental cost of using more purchased resources. Similar behaviour in the mountainous region of the Atlantic Forest was observed by Ortega (2001).

Emergy exchange Ratio (EER)

The emergy exchange ratio shows that the transaction of the ecological production systems

(1.23) and citrus (1.91) do not receive a fair price. The received emergy by the transaction demonstrates that the systems export more emergy that the one received through the payment of the products. The cattle (0.47), sylvopastoral (0.43), vegetables fruit (0.61), leaf (0.92) mixed (0.61) give less energy to the buying system than to the producing system.

Conclusions

a) The horticultural systems use more manpower in comparison to the other systems; the ones that use less are the cattle systems. Due to the forest handling and agroforestry inside the same property, the ecological systems in general, make low use of manpower per hectare. But if it takes exclusively into account the agricultural area this option uses more manpower than that of all other studied systems.

b) The horticultural intensive systems in general obtain better net income and are also the most dependent in inputs coming from the economy and for this reason more unstable. They contribute also less to the economy of the region, because of their low use of renewable resources.

c) Cattle production is one of the most important components of agriculture in Teresópolis, being the main consumer of natural resources all together. Cattle production contributes to the degradation of resources, namely, land degradation, water scarcity and pollution, global warming, and diminishing biodiversity.

d) The cattle system causes bigger environmental damage and they have the smallest yield per hectare in economic and energy terms. Although these do not depend on resources coming from the economy they use many non-renewable resources. The erosion is the most important factor in terms of non-renewable resources. In economic terms this soil loss represents a very high value.

e) The vegetable systems had large amounts of energy invested in irrigation, fertilizers and fuels, and the cattle systems use great quantities of non-renewable resources, leading to a loss of autonomy of producers in relation to technology and prices fixed abroad. The ecological systems demonstrated potential gains in sustainability by reducing the energy devoted to these inputs. Because large amounts of non-renewable energies are required to supply water and nutrients to fields, finding methods to reduce these inputs has great potential to increase the sustainability and decrease the environmental loading of agricultural production.

f) The largest value of sustainability corresponds to the ecological systems in ecological

terms and also it is the only one that has the capacity to save capital in form of biomass in the system. These systems use fewer resources from economy and more natural renewable resources, which guarantee its sustainability. They ensure the survival of the producer throughout the time and the preservation of the biodiversity.

g) The substitution of the cattle systems by any other agricultural or forest system represents economic and environmental clear gains. The best options are the biological, agroforestry and forest systems.

h) The citrus systems is not the best, but could be also a good alternative, because the low investment rate, and its great aptitude to grow in hills. The use of renewable resources from this system is comparable to the vegetable systems. The use of material and services are very low, the transformity and renewability are better than the vegetable systems.

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