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Conversion of Sugarcane Shoots and Leaves into Reducing Sugars by Pretreatment and Enzymatic Hydrolysis

Teerapatr Srinorakutara[1], Suthkamol Suttikul[1] and Pornpattra Srinorakutara[2]

1. Department of Energy Technology, Thailand Institute of Scientific and Technological Research, Pathum Thani 12120, Thailand

2. Department of Food Technology, Thailand Institute of Scientific and Technological Research, Pathum Thani 12120, Thailand

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Abstract: Sugarcane shoots and leaves consist of 38% cellulose, 30.6% hemicellulose and 12.8% lignin on dry solid (DS) basis and have the potential to serve as low cost feedstocks for ethanol production. The pretreatment and enzymatic hydrolysis conditions include particle size, alkali (NaOH)/dilute acid (H_2SO_4) pretreatment, chemical and substrate concentrations, temperature, autoclaving time for pretreatment, enzyme concentration, pH and temperature for hydrolysis varied were evaluated for conversion of sugarcane shoots and leaves cellulose and hemicellulose to reducing sugar. The optimum conditions were accomplished by using 14% w/v DS of 0-10 mm sugarcane shoots and leaves in particle size, pretreated with 1.5% w/v of dilute sulfuric acid at 121 ℃, 15 lbs/in^2 for 15 min and enzymatic saccharification using 40 FPU/g DS cellulose at 50 ℃ and pH 5. After incubating at 160 rpm for 12 hrs, 59 g/L or 386.38 mg/g DS of reducing sugar and 50.69% saccharification were obtained.

Key words: Cellulosic biomass, dilute acid pretreatment and enzymatic hydrolysis, sugarcane shoots and leaves, ethanol production.

1. Introduction

As conventional energy sources (oil, coal and gas) are not renewable, utilization of biomass to obtain alternatives to petroleum products can reduce the energy crisis. The basic raw materials (sugars, starches and celluloses) used for the fermentative production of ethanol, cellulosic materials are receiving major research attention because of their abundant availability, mostly as waste materials [1].

Sugarcane shoots and leaves (agroresidues), obtained after harvesting the crop, are used neither as animal feed (because of the rough texture of shoots and leaves), nor as fuel. Burning this biomass after harvest causes pollution problems, and the utilization of this biomass would provide a new source of energy together with aiding pollution abatement [2].

The conversion of lignocellulosic biomass to ethanol is, however, more challenging than corn due to the complex structure of the plant cell wall. Pretreatment is required to alter the structural and chemical composition of lignocellulosics biomass to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars [3].

The objective of this study was to find the optimum operating conditions of pretreatment and enzymatic hydrolysis steps to produce reducing sugars from sugarcane shoots and leaves by pretreatment and saccharification for ethanol production.

2. Materials and Methods

2.1 Substrate and Size Reduction

Sugarcane (*Saccharum officinarum* Linn) shoots and leaves were collected from sugarcane farm in

Corresponding author: Teerapatr Srinorakutara, Ph.D., research field: biochemical engineering. E-mail: teerapatr@tistr.or.th.

Angthong province, Thailand. Prior to use, sugarcane shoots and leaves were passed through vegetable chopper to cut into small pieces and dried before use. Dried biomass was ground into smaller size and passed through screener to separate into particle sizes of 0-5, > 5-10, > 10-20 and > 20 mm.

2.2 Pretreatment

Sugarcane shoots and leaves of 10 g at different particle size of 0-5, > 5-10, > 10-20 and > 20 mms were transferred to 250 mL duran bottle. In this study 1% w/v H_2SO_4 [3-8] and 1% w/v NaOH [3, 9-11] were compared as the pretreatment chemicals. Hundred milliliters of pretreatment chemicals were added to biomass and mixed well. The mixtures were then autoclaved at 121 ℃, 15 lbs/in^2 for 15 min.

2.3 Enzymatic Hydrolysis

Accellerase 1000 (derived from *Trichoderma reesei*; Genencor, Rochester, NY, USA) containing cellulase activity 265 FPU/mL was used as cellulase enzyme in saccharification step.

After cooled down, the mixtures were adjusted pH to 5 and 20 FPU/g DS Accellerase 1000 were added. The reaction was conducted at 50 ℃ in shaking incubator at 160 rpm for 24 hrs. Aliquots of 500 μL were periodically taken and centrifuged. The supernatants were analyzed for reducing sugars [12].

The effect of substrate concentration for pretreatment on reducing sugar production from sugarcane shoots and leaves was investigated using the same methods as described above. The different amounts of sugarcane shoots and leaves (10, 12, 14, 18, and 20% w/v) were used.

The effect of pretreatment chemicals concentration on reducing sugar production from sugarcane shoots and leaves was investigated using the same methods as described above. The different concentrations of pretreatment chemicals (0.5, 0.75, 1, 1.5, and 2% w/v) were used.

The effect of pretreatment temperature on reducing sugar production from sugarcane shoots and leaves was investigated using the same methods as described above. The different temperatures (110, 115, 121, and 125 ℃) were used.

The effect of autoclaving time for pretreatment on reducing sugar production from sugarcane shoots and leaves was investigated using the same methods as described above. The different autoclaving times (10, 15, 30, 45, and 60 min) were used.

The effect of enzyme concentration for hydrolysis on reducing sugar production from sugarcane shoots and leaves was investigated using the same methods as described above. The different enzyme concentrations (10, 20, 40, 60, 80, and 100 FPU/g DS) were used.

The effect of pH for hydrolysis on reducing sugar production from sugarcane shoots and leaves was investigated using the same methods as described above. The different pHs (4.0, 4.5, 5.0, 5.5 and 6.0) were used.

The effect of temperature for hydrolysis on reducing sugar production from sugarcane shoots and leaves was also investigated using the same methods as described above. The different temperatures (40, 50, and 60 ℃) were used.

Reducing sugars were analyzed using dinitrosalicylic acid reagent comparing with the standard glucose [13].

All experiments were conducted in triplicate.

3. Results and Discussion

3.1 Composition of Sugarcane Shoots and Leaves

Sugarcane shoots and leaves were analyzed for their compositions (Table 1). The levels of major components of dried sugarcane shoots and leaves found were 38.0% cellulose, 30.6% hemicellulose, and 12.8% lignin. The results showed that these raw materials had potential for reducing sugar production by conversion from their cellulose components.

3.2 The Effect of Pretreatment Chemicals and Particle Size on Reducing Sugar Production

The phenomenon of sugarcane shoots and leaves at different sizes after pretreatment and after enzymatic hydrolysis is shown in Figs. 1 and 2.

Lignocellulosic biomass requires pretreatment, mainly because the lignin in plant cell walls forms a barrier against enzyme attack. An ideal pretreatment reduces the lignin content and crystallinity of the cellulose and increases surface areas [1].

In this study, two pretreatment chemicals (H_2SO_4 and NaOH) were compared. The enzymic hydrolysis of substrate treated with H_2SO_4 was found to be superior to alkali treatment.

The effect of pretreatment chemicals of sugarcane shoots and leaves on reducing sugars yield is shown in Table 2 and Fig. 3. At different sizes of biomass

Table 1 The compositions of sugarcane shoots and leaves.

Composition	Percentage (DW)
Holocellulose	68.6
Cellulose	38.0
Hemicellulose	30.6
Lignin	12.8

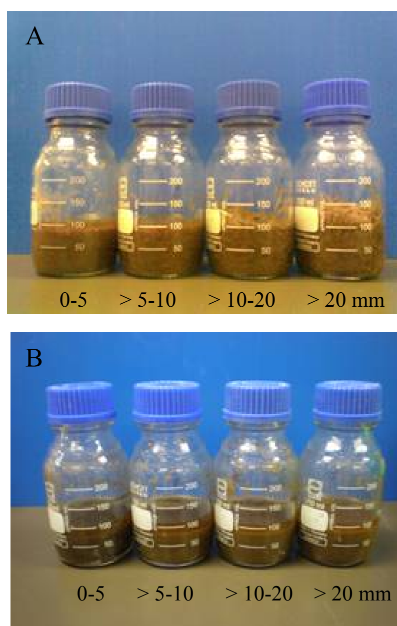

Fig. 1 Sugarcane shoots and leaves at different particle sizes after pretreatment with 1% w/v H_2SO_4 at 121 ℃ for 15 min (A) and after hydrolysis with Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs (B).

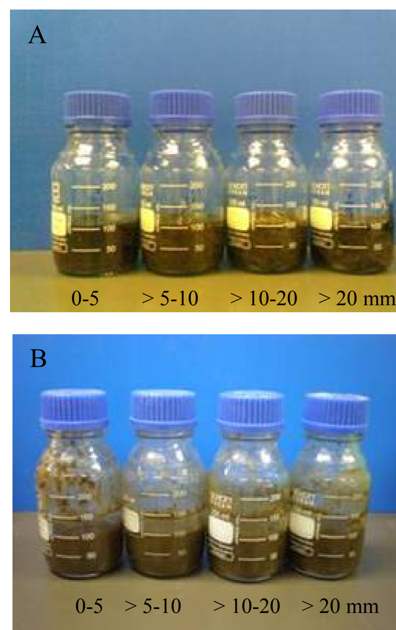

Fig. 2 Sugarcane shoots and leaves at different particle sizes after pretreatment with 1% w/v NaOH at 121 ℃ for 15 min (A) and after hydrolysis with Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs (B).

pretreated with 1% w/v H_2SO_4 diluted acid giving 35-37 g/L reducing sugars was higher than the same size of biomass pretreated with 1% w/v NaOH alkali giving 31-33 g/L reducing sugars.

The yield of reducing sugars, cost of chemicals reagent and the convenience in pH adjustment process were considered. The use of 1% w/v H_2SO_4 diluted acid for pretreatment was selected in all further experiments.

As shown in Table 2 and Fig. 3, the different particle sizes of biomass (0-5, 5-10, 10-20 and > 20 mm) after pretreatment with diluted acid or alkali had no significant effect on reducing sugar production. They gave 35.16, 36.35, 37.11 and 37.60 g/L by using diluted acid pretreatments and 31.99, 33.78, 32.77 and 32.08 g/L by using alkali pretreatments respectively. Similarly, the percent of saccharification obtained in the diluted acid pretreatments were 48.27, 49.70, 50.75 and 51.41% as the alkali pretreatments obtained were 40.99, 44.39, 43.23 and 41.81% respectively. The results showed that the particle size of biomass did not affect the reducing sugar yield. With regard to the practical process of transportation material through

Table 2 The reducing sugars obtaining after pretreatment and hydrolysis at different particle size of sugarcane shoots and leaves by using Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Pretreatment chemicals	Particle size (mm)	Reducing sugars (g/L)	Saccharification (%)
1% (w/v) H_2SO_4	0-5.0	35.16 ± 0.16	48.27 ± 0.20
	> 5.0-10.0	36.35 ± 0.95	49.70 ± 1.31
	> 10.0-20.0	37.11 ± 0.66	50.75 ± 0.91
	> 20.0	37.60 ± 0.15	51.41 ± 0.20
1% (w/v) NaOH	0-5.0	31.99 ± 0.08	40.99 ± 0.20
	> 5.0-10.0	33.78 ± 0.40	44.39 ± 1.14
	> 10.0-20.0	32.77 ± 0.62	43.23 ± 0.83
	> 20.0	32.08 ± 0.40	41.81 ± 0.47

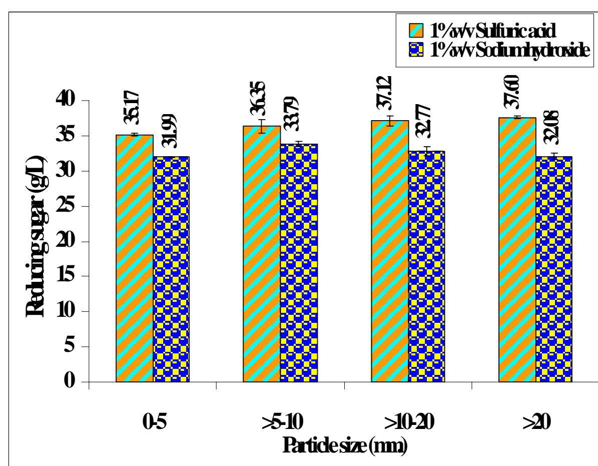

Fig. 3 The reducing sugars obtained after pretreatment and hydrolysis at different particle sizes of sugarcane shoots and leaves by using Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

pipe line in plant, the particle size of biomass 0-10 mm was appropriate to be used in all further experiments.

3.3 The Effect of Substrate Concentration on Reducing Sugar Production

The effect of substrate concentration on reducing sugar yield after pretreatment 0-10 mm sugarcane shoots and leaves in particle size with 1% H_2SO_4 by autoclave at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (20 FPU/ g DS) at 50 ℃, pH 5 for 24 hrs is illustrated in Table 3 and Fig. 4. The substrate concentrations of 10, 12, 14, 18 and 20% w/v increased in the reacting mixtures would raise the reducing sugars yield. In particular, when the concentration of substrate increased from 10 to 12, 14 and 16% w/v, the yield of reducing sugars would raise from 36.62 to 43.04, 49.29 and 52.67 g/L respectively. An increase in substrate concentration between 18 and 20% w/v would limit the rate of saccharification, because the mixtures were associated difficulties giving the yield of reducing sugar 53.18 and 55.51 g/L respectively.

An increase in the substrate concentration adversely affected the saccharification. The substrate concentration increased from 10 to 12, 14, 16, 18 and 20% w/v would decrease the percent of saccharification to 48.32, to 45.61, 43.82, 39.74, 34.25 and 31.13%, respectively as shown in Table 3.

These results including the appropriateness of mixture viscosity, the substrate concentration of 14% w/v were selected to use in all further experiments.

3.4 The Effect of Pretreatment Chemicals Concentration on Reducing Sugar Production

The effect of pretreatment chemicals (H_2SO_4) concentration of 0.5, 0.75, 1.0, 1.5 and 2.0% w/v on 0-10 mm biomass in particle size was illustrated in Table 4 and Fig. 5. Acid concentration increased in the reacting mixture from 0.5 to 0.75, 1.0, 1.5 and 2.0% w/v led to an increase in reducing sugars from 23.32 to 30.96, 45.32, 53.14 and 54.86 g/L respectively.

Similarly, the acid concentration in the reacting mixture increasing from 0.5 to 0.75, 1.0, 1.5 and 2.0% w/v also led to an increase in the percent saccharification from 19.4 to 26.86, 40.13, 48.28 and 50.89% respectively. The results showed that acid

Table 3 The reducing sugars obtaining after pretreatment 0-10 mm sugarcane shoots and leaves in particle size at different amount of substrate with 1% w/v H_2SO_4 at 121 ℃ for 15 min and hydrolysis by using Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Substrate concentration (% w/v)	Reducing sugar (g/L)	Saccharification (%)
10	36.62 ± 0.79	48.32 ± 1.04
12	43.04 ± 0.35	45.61 ± 0.38
14	49.29 ± 0.82	43.82 ± 0.67
16	52.67 ± 0.82	39.74 ± 0.99
18	53.18 ± 0.93	34.25 ± 0.66
20	55.51 ± 0.58	31.13 ± 0.50

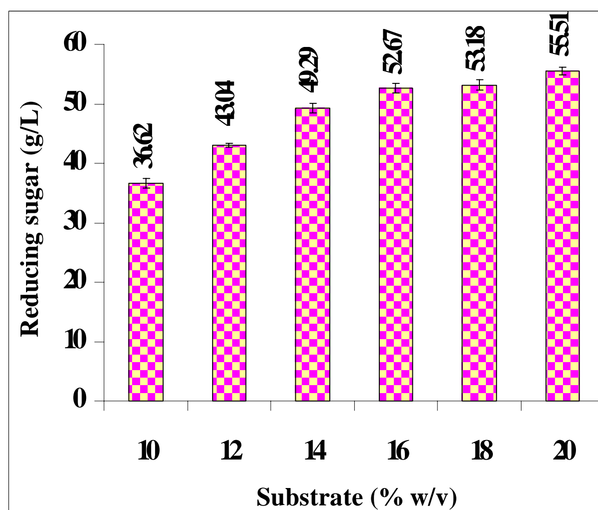

Fig. 4 The reducing sugars obtaining after pretreatment 0-10 mm sugarcane shoots and leaves in particle size at different amount of substrate with 1% w/v H_2SO_4 at 121 ℃ for 15 min and hydrolysis by using Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Table 4 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with different concentrations of H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Chemicals concentration (% w/v)	Reducing sugars (g/L)	Saccharification (%)
0.5	23.32 ± 0.24	19.40 ± 0.33
0.75	30.96 ± 0.28	26.82 ± 0.11
1.0	45.32 ± 0.27	40.13 ± 0.24
1.5	53.14 ± 0.16	48.28 ± 0.14
2.0	54.86 ± 0.13	50.89 ± 0.44

concentration of 1.5 and 2.0% w/v had no significant effect on both reducing sugar and the percent of saccharification. From these results, economics and waste water minimization considered, 1.5% w/v H_2SO_4 was therefore selected for pretreatment of biomass.

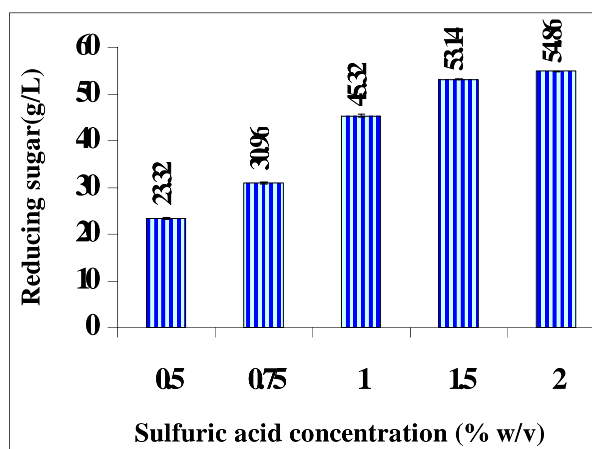

Fig. 5 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with different concentrations of H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

3.5 The Effect of Pretreatment Temperature on Reducing Sugar Production

The effect of pretreatment at different temperatures of 110, 115, 121 and 125 ℃ was illustrated in Fig. 6 and Table 5. Temperature of pretreatment increasing from 110 to 115, 121 and 125 ℃ led to an increase in reducing sugars from 45.63 to 50.58, 54.81 and 57.61 g/L or 288.04 to 323.34, 354.67 and 372.82 mg/g DS respectively. Similarly, the percent of saccharification also increased to be 43.04 to 47.72, 51.49 and 53.02% respectively as temperatures were risen. This might be that the stronger reaction can reduce more crystallinity of cellulose leading to an increase in more surface areas. However, the reducing sugars achieved from pretreatment at 121 and 125 ℃ were not different. To reduce energy consumption, autoclaving the reaction mixtures at 121 ℃ was selected as optimum temperature of pretreatment.

3.6 The Effect of Autoclaving Time on Reducing Sugar Production

The effect of autoclaving times of 10, 15, 30, 45 and 60 min is shown in Fig. 7 and Table 6. Autoclaving times increased from 10 to 15 min led to an increase in reducing sugars of 51.32 to 57.70 g/L or 344.20 to 386 mg/g DS respectively. The % saccharifications were

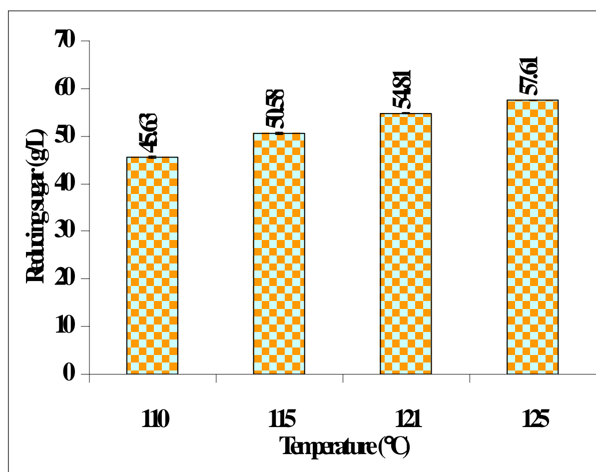

Fig. 6 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at different temperatures and hydrolysis by Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Table 5 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves particle size with 1.5%H_2SO_4 at different temperatures and hydrolysis by Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Temperature (℃)	Reducing sugars (g/L)	Saccharification (%)
110	45.63 ± 0.18	43.04 ± 0.16
115	50.58 ± 0.27	47.72 ± 0.25
121	54.81 ± 0.18	51.49 ± 0.51
125	57.61 ± 0.02	53.02 ± 0.02

also increased from 50.17 to 56.41%. As extension of the reaction time to 30 min or more had no significant effect on saccharification, 15 min was therefore selected as the optimum autoclaving time.

3.7 The Effect of Enzyme Concentration for Hydrolysis on Reducing Sugar Production

During initial experiments, Accellerase 1000 was used at 20 FPU/g DS. To identify a suitable enzyme concentration for saccharification, experiments with different concentrations of Accellerase 1000 (10-100 FPU/g DS) were performed and maximum conversion was achieved with 100 FPU cellulose at 12 hrs shown in Fig. 8 and Table 7. An increase in the enzyme concentration from 40 to 100 FPU would increase reducing sugars and the percent of saccharification

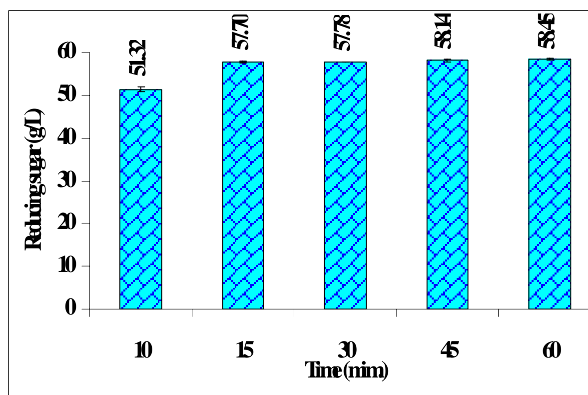

Fig. 7 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4, 121 ℃ at different autoclaving time and hydrolysis by Accellerase 1000 (20 FPU/g DS) at 50℃, pH 5 for 24 hrs.

Table 6 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4, 121 ℃ at different autoclaving times and hydrolysis by Accellerase 1000 (20 FPU/g DS) at 50 ℃, pH 5 for 24 hrs.

Autoclaving time (min)	Reducing sugars (g/L)	Saccharification (%)
10	51.32 ± 0.56	50.17 ± 0.55
15	57.70 ± 0.22	56.41 ± 0.22
30	57.78 ± 0.06	56.49 ± 0.05
45	58.14 ± 0.36	56.84 ± 0.36
60	58.45 ± 0.32	57.14 ± 0.31

only 4.9 g/L and 8%, respectively, as the use of such high enzyme concentrations might not be justified economically. Therefore 40 FPU/g DS of Accellerase 1000 was selected as optimum enzyme concentration to use for further experiments. An increase in the rate of saccharification at high concentration of cellulose might be due to the increase in rates of *trans*-glycosylation reaction [14]. The optimum reaction time was 12 hrs as extension of the reaction time to 24 hrs or more had no significant effect on saccharification.

3.8 Effect of pH for Enzymatic Hydrolysis on Reducing Sugar Production

The effect of pH for enzymatic hydrolysis on reducing sugars was illustrated in Fig. 9 and Table 8. At pH 5.0 there were maximum yield of reducing

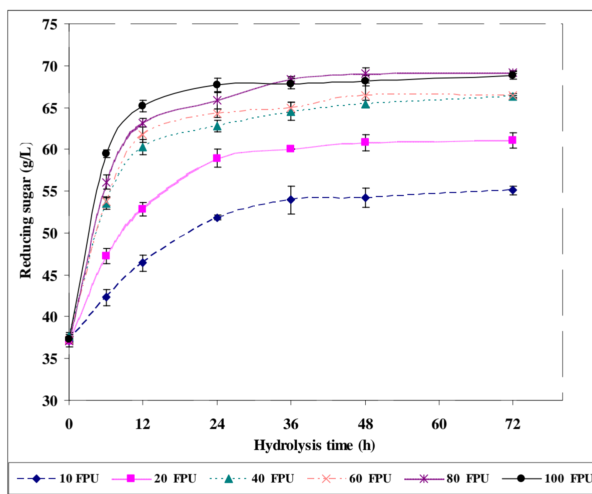

Fig. 8 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 at different enzyme concentrations at 50 ℃, pH 5 for 24 hrs.

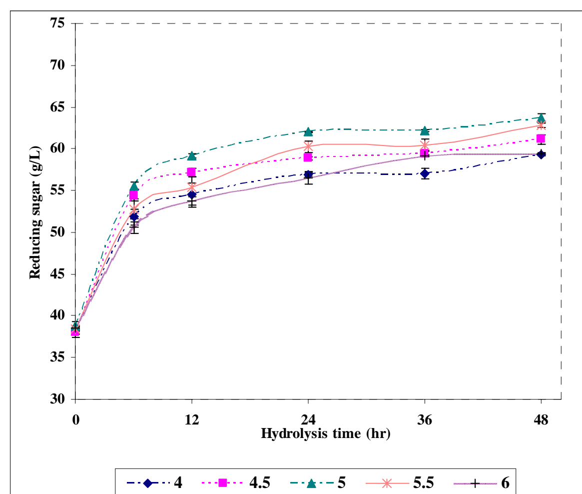

Fig. 9 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (40 FPU/g substrate), 50 ℃ at different pHs for 24 hrs.

Table 7 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at 121 ℃, 15 min and hydrolysis by Accellerase 1000 at different enzyme concentrations at 50 ℃, pH 5 for 24 hrs.

Enzyme concentration (FPU/g DS)	Reducing sugars (g/L)	Saccharification (%)
10	46.41 ± 0.98	40.36 ± 0.86
20	52.87 ± 0.81	46.16 ± 0.41
40	60.27 ± 0.89	52.82 ± 0.84
60	61.77 ± 0.90	56.07 ± 1.08
80	63.18 ± 0.52	58.86 ± 0.49
100	65.17 ± 0.65	60.71 ± 0.60

sugars (59.14 g/L) and the percent of saccharification (50.61%), although other pHs in the range 4.0-6.0 did not affect the yield comparing to pH 5.0. The slight decrease in the saccharification at lower pH values was favorable to SSF process. The optimum pH for yeast growth was normally between 5.0 and 5.5 as a decrease in pH below 4.0 wound take place during fermentation. These results showed that such a change in pH during fermentation did not bring about a large effect on the total efficiency of saccharification.

3.9 Effect of Temperature for Enzymatic Hydrolysis on Reducing Sugar Production

The effect of temperature at 40, 50 and 60 ℃ for

Table 8 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (40 FPU/g DS), 50 ℃ at different pHs for 24 hrs.

pH	Reducing sugars (g/L)	Saccharification (%)
4.0	54.55 ± 1.29	46.12 ± 1.33
4.5	57.18 ± 0.50	47.94 ± 0.41
5.0	59.14 ± 0.26	50.61 ± 0.89
5.5	55.43 ± 1.22	47.24 ± 0.77
6.0	53.73 ± 0.74	45.43 ± 1.06

Table 9 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (40 FPU/g DS), pH 5 at different temperatures for 24 hrs.

Temperature (℃)	Reducing sugars (g/L)	Saccharification (%)
40	51.88 ± 0.32	43.50 ± 0.49
50	59.00 ± 0.49	50.69 ± 0.49
60	51.69 ± 0.82	42.99 ± 0.42

enzymatic hydrolysis on reducing sugars was shown in Fig. 10 and Table 9. The optimal temperature for enzymatic hydrolysis was 50 ℃ giving 59 g/L of reducing sugars and 50.69% saccharification at 12 hrs. In particular, temperatures at 40 ℃ and 60 ℃ caused drastic decrease in the rate of saccharification giving

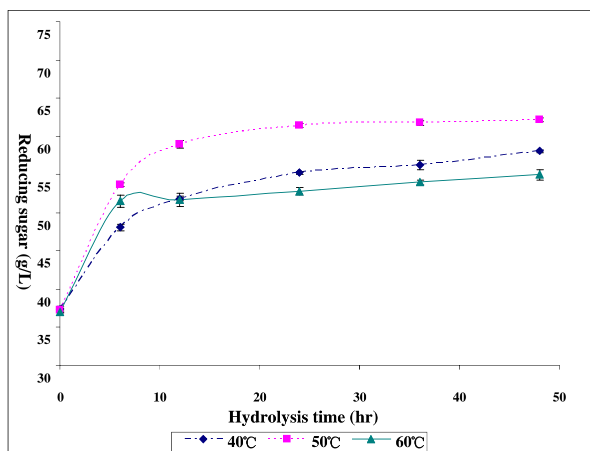

Fig. 10 The reducing sugars obtaining after pretreatment 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size with 1.5% H_2SO_4 at 121 ℃ for 15 min and hydrolysis by Accellerase 1000 (40 FPU/g DS), pH 5 at different temperatures for 24 hrs.

51.88 and 51.69 g/L of reducing sugars and 43.50 and 42.99% saccharification respectively.

4. Conclusion

The optimum conditions to produced reducing sugars from sugarcane shoots and leaves were two steps consisting of pretreatment and enzymatic hydrolysis. The pretreatment step was conducted by using 14% w/v of 0-10 mm sugarcane shoots and leaves in particle size, pretreated with 1.5% w/v of H_2SO_4 by autoclave at 121 ℃, 15 lbs/ in^2 for 15 min. The enzymatic hydrolysis step was conducted by hydrolysis the pretreated substrate with Accellerase 1000 (40 FPU/g DS) at 50 ℃, pH 5. After 12 hrs of incubation, the 59 g/L or 386.38 mg/g DS of reducing sugar and 50.69% saccharification were obtained.

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Application Prospects for the Innovation of Defined Fungal Starter in Rice Wine Fermentation

Ngo Thi Phuong Dung and Huynh Xuan Phong

Biotechnology Research and Development Institute, Can Tho University, Can Tho City 92000, Vietnam

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Abstract: The feasibility of pilot-scale manufacture of defined fungal starter and its application in rice wine production from different local starchy materials were investigated. Starter consisting of *Amylomyces rouxii* and *Saccharomyces cerevisiae* gave high performance in winemaking when prepared in conditions of rice flour 80% and cassava flour 20% with 4 incubation days. The starter level at 20% was favourably employed for manufacture with the initial amount of 10 kg mixed-flours. Dry starter granules which were vacuum packed could adapt ambient temperature (approx. 28-32 ℃) during 8 months of storage. The defined starter performed as superior inoculum for winemaking from different agricultural starchy resources. The undesirable bacteria were found at approx. 2 Log CFU/g of dry starter. By morphology, biochemical and physiological growth and the genetic partial 16S analyses, three bacterial isolates were characterized as *Bacillus subtilis/amyloliquefaciens* which may contaminate food but not cause food poisoning and not considered as a human pathogen.

Key words: Defined starter, rice wine, alcoholic fermentation, pilot-scale manufacture, undesirable bacteria.

1. Introduction

The principle of rice wine manufacture consists of the saccharification of steamed rice starch by fungal enzymes and the simultaneous or subsequent alcoholic fermentation by yeasts from traditional starter tablets [1, 2]. The limited knowledge about the microbiological composition of traditional starters in relation to their performance in the fermentation poses an obstacle to industrial development, and thus the development of defined starters containing mixed pure cultures is a priority in food microbiology and technology research. Advantages of defined mixed starter cultures have been described in Refs. [3-5]. Rice wine starters are generally composed of essential and non-essential microorganisms, the presence of moulds and yeasts however is considered essential for this type of fermentation [2, 6].

In Vietnam, each local producer may have a different way of starter production, depending on available ingredients and local custom and preferences. Following the same long-term research project, the recent studies [7-9] have addressed the problem of poor and variable quality of traditional starter tablets, by understanding and quantifying the impact of microflora in these starters, concentrating on mycelial fungi and yeasts, and by assessing the option of preparing stable mixed cultures of selected compatible strains. These studies have successfully identified fungal cultures isolated from various commercial traditional rice wine starters in Vietnam and developed a laboratory-scale process to formulate defined mixed-culture starter granules that produced wine with superior flavours and overall acceptability.

Besides, the raw ingredients for starter manufacture contain a diversity of microorganisms, including spore-forming bacteria that could have survived the heat treatment, some of these genera have been reported as predominant bacteria in fermented cassava

Corresponding author: Ngo Thi Phuong Dung, Ph.D., research fields: food microbiology, food fermentation, food safety and quality. E-mail: ntpdung@ctu.edu.vn.

dough [10]. Although a certain number of undesirable bacteria was found in fermented starter dough [7], they gave no clear competitive influences on the growth and the contribution of fungal cultures during the saccharification and the alcoholic fermentation process, and neither expect nor exclusively being interested in mesophillic bacteria, it is obviously useful to characterize these bacterial isolates, particularly for the safety before making a transfer of the new product into practice.

As a logical continuation with the ultimate aim for the application of experimental defined starter processing into practice, this project investigated the manufacture of defined starter granules at pilot-scale trial and their performance in winemaking from different agricultural starchy materials. Factors affecting the storage stability of dry starter and the purification and identification of undesirable bacteria were also included.

2. Materials and Methods

2.1 Culture and Preparation of Strains

The mould of *Amylomyces rouxii* (CBS 111757; LU 2043) and the yeast of *Saccharomyces cerevisiae* (LU 1250) were employed. These strains were isolated [9] from Vietnamese rice wine starters and selected [8] for their superior ability to degrade starch and accumulate alcohol, respectively.

The cultures were grown on slants of Malt Extract Agar (MEA) at 30 ℃ for 5 days (mould) or 2 days (yeast). A suspension of the growing microorganisms was made by adding 5 mL of sterile physiological salt solution (0.85% NaCl) to each slant. The biomass was scraped off the agar by means of inoculating wire.

2.2 Effects of Ingredients and Incubation Time on Starter Processing

A factorial design (2 factors at 3 levels) was used including ingredients (rice flour 100%, rice flour 80% + cassava flour 20%, rice flour 80% + maize flour 20%), and incubation time (3, 4 and 5 days). Each treatment had triplicates. By modifying the appropriate time for the laboratory-scale preparation of defined fungal starter granules [7], the processing was tested as follows: 300 g of ingredients that had been heated at 100 ℃ overnight were prepared in sterile polypropylene bag. The heated ingredients were cooled to 35-40 ℃ and the moisture content at 40% w/w was prepared with the sterile solution containing glucose 1%, lactose 1%, $(NH_4)_2SO_4$ 0.2%, $MgSO_4$ 0.05%, $CaSO_4$ 0.02%, KH_2PO_4 0.1%, acid glutamic 0.05%. A mould suspension 10% v/w of 10^6 spores/mL and a yeast suspension 1% v/w of 10^8 cells/mL were inoculated, mixed well and incubated at 30 ℃. The fermented starter dough was subsequently transformed into granules and dehydrated in a ventilated oven (FED 115, Binder, Germany) on perforated drying trays at 42 ℃ for 17 hours. The dry samples were used as inoculum in the winemaking for required analyses.

2.3 The Feasibility of Pilot-scale Starter Manufacture

As a pilot-scale testing trial the starter preparation was prepared with the initial amount of ingredients at 10 kg per batch. In this procedure, the most favourable conditions of ingredients and incubation time from the previous experiment (2.2) were selected for starter preparation. The final product of starter granules obtained in the starter manufacture was used as stock inoculum. The inoculum levels were tested at 10%, 20% and 30% w/w of ingredients. Each treatment had triplicates.

2.4 Storage Shelf-life of Dry Starter Granules

In the manufacture processing of starter, the final dry starter granules were vacuum packed in sterile polypropylene bags with 0.06 mm thickness using a “Vacupack plus” machine (Krups, type 380, CE, z260590, P.R.C.) and stored at ambient temperature (approx. 28-32 ℃). Analyses of the fungal performance in the winemaking were done after 0, 2, 4, 6 and 8 months of storage.

2.5 Purification and Identification of Bacterial Contaminants

In the production of mixed-culture starter granules, the final product was suspended with sterile physiological salt solution 0.85% and pour-plated into Plate Count Agar (PCA) supplemented with natamycin, incubated at 30 ℃ for 1-4 days, and colonies were isolated and purified on PCA. The colonies that appeared after incubation were counted, calculated as colony forming units (CFU) and expressed as Log CFU/g. Pure isolates of bacteria were stored on MEA. The identification activity was carried out with the support of CBS Institute Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands), including following methods: morphological examination, anaerobic growth on TSA, assimilation of carbonate nitrogen compounds by using Biolog GP plates (STAG), and DNA extraction, amplification and sequencing by using DYE-ET terminator cycle sequencing (Amersham Biosciences). Sequence similarity searches were performed with the BLAST program as supported by the NCBI website (http://www.ncbi.nlm.nih.gov/blast).

2.6 Assessment of Defined Starter Granules in the Winemaking Fermented from Different Agricultural Starchy Materials

The ability of defined starter and its validation in comparison with commercial starters were assessed in the winemaking from different agricultural starchy materials. There were two factors including starter inoculation (defined fungal starter, Hai Anh Quang starter and Phuc Hung starter) and starchy materials (purple glutinous rice, Phu Tan glutinous rice, Huong Lai rice and Huyet Rong rice). Each treatment had triplicates.

2.7 Analytical Methods

2.7.1 Assessment of Starter Performance in Winemaking

Fifty grams of agricultural starchy material and 60 mL of distilled water in a 250 mL conical flask covered by a cotton plug, were soaked for 4 hours at room temperature. After soaking they were steamed in an autoclave for 1 hour at 100 ℃. The steamed rice paste was cooled to 30-40 ℃, then inoculated and mixed well with starter 2%. After solid-state fermentation during 3 days at 30 ℃, 70 mL of sterile water was added to the moulded mass to allow for submerged alcoholic fermentation during 4 days at 30 ℃ under anaerobic conditions by replacing the cotton plug with a water lock. Samples were harvested for required analyses.

2.7.2 Chemical Analyses

The pH was measured by a digital pH meter (Sartorius, PB-20, Germany). Total dissolved solids content (mainly sugar) of saccharified liquid was estimated by measuring ^{o}Brix with a manual refractometer (FG 103/113, Euromex Holland). Glucose contents were determined by glucose oxidase test kit (Megazyme, GLC 9/96). Total alcohol content was determined by the distillation method [11]. Biomass was determined by the dry matter method.

2.7.3 Statistical Analysis

Experimental data were analysed statistically using Statgraphics Plus Version 5, Manugistics, Inc., Rockville, USA.

3. Results and Discussion

3.1 Effects of Ingredients and Incubation Time on Defined Starter Processing

By simplifying the appropriate time for the laboratory-scale preparation of defined fungal starter granules [7], the present process was investigated with the aim to pave the way for transferring the know-how of preparation and application of experimental starter to pilot-scale manufactures into practice. As a cheap and suitable supplement for a good combination with rice flour ingredient that had been mainly employed in the production of alcoholic starters in Vietnam [6], cassava flour and maize flour were tested in turn in the conditions of different incubation time at 3, 4 and 5 days. In all treatments, the fermented starter dough was

obviously adhesive with the mycelium increased gradually during the incubation and fully spread up to 4 days of incubation. However, after 5 days of incubation the mycelium was vanished and the fermented dough became inadhesive. After dehydration the starter granules were harvested with the moisture content of 7.8%. They were employed as inoculums in the winemaking for the assessment of their ability. All starter granules achieved normal and successful wine fermentation from purple glutinous rice with adequate performance during the solid-state fermentation and alcoholic fermentation.

During the incubation, the pH identically rapidly decreased in all treatments and reach in a range of 4.6-5.0 after the alcoholic fermentation, as compared to a pH of 6.02 of the uninoculated control. This pH range was also indicative of the purity and ability of the fungal cultures, since in cultures contaminated with (acidifying) bacteria, the pH usually became considerably lower, in the range of pH 3.0-3.5. Table 1 showed the results of winemaking performance in defined starter granules of different ingredients and incubation time.

During three days of incubation for the saccharification stage, the mycelium appearance and the liquefaction were increasingly obvious in all treatments. The glucose production was also achieved, of which the treatment including rice flour 80% and cassava flour 20% with 4 days of incubation gave the significantly highest content of glucose (20.4% w/v). Actually, the glucose produced during the solid-state fermentation could reach more higher level in all treatments; however, the inoculum comprised mixed fungal pure cultures of the mould *Amylomyces rouxii* and the yeast *Saccharomyces cerevisiae*, therefore as soon as glucose produced by mould, it was consumed and fermented by yeast into ethanol. Concomitantly, in the alcoholic fermentation stage, the rapidly high fermentation rate was clearly observed after 1 day of incubation and the high alcohol contents in the final rice wine product were obtained. In addition, the species *Amylomyces rouxii* belonged to genera of the Zygomycetes and was confined to the order Mucorales, and the mould in this order could produce alcohol under low oxygen conditions [12]. It could support to explain for the rapid and strong fermentation signal at the first beginning time of the alcoholic fermentation.

For the assessment of the fermentative capacity, the treatment including rice flour 80% and cassava flour 20% with 4 days of incubation was also found to give the highest content of ethanol (15% v/v). To combine with the results of the defined starter performance in the saccharification process, this treatment was indicated as favourable conditions for the preparation

Table 1 Winemaking performance in defined starter granules of different ingredients and incubation time.

Ingredient	Incubation time (d)	Results of solid-state fermentation								Results of alcoholic fermentation					
		Mycelium appearance[1] (per d)			Saccharified liquid appearance[2] (per d)			Glucose		Fermentation rate[3] at different time (h)				Alcohol	
		1	2	3	1	2	3	% w/v	SD[4]	24	48	72	96	% v/v	SD
Rice flour 100%	3	+	++	+++	-[5]	+	++	18.18[6] bcd[7]	0.15	10	14	2	0	13 bc	1
	4	+	++	+++	-	++	+++	16.79 ef	0.19	15	16	2	1	13.67 ab	0.57
	5	+	++	+++	-	+	++	17.87 cd	0.71	11	14	1	1	11 d	1
Rice flour 80% + cassava flour 20%	3	+	++	+++	-	+	++	19.01 b	0.73	13	12	1	1	13.33 ab	1.15
	4	+	++	+++	-	++	+++	20.40 a	0.46	19	17	2	1	15 a	1
	5	+	++	+++	-	+	++	17.65 de	0.5	14	11	1	1	12.33 bcd	0.57
Rice flour 80% + maize flour 20%	3	+	++	+++	-	+	++	16.66 ef	0.6	13	15	1	1	12.33 bcd	1.52
	4	+	++	+++	-	++	+++	18.92 bc	1.14	18	15	2	1	14 ab	0
	5	+	++	+++	-	+	++	16.26 f	0.54	12	13	3	1	11.33 cd	1.52

[1] levels of mycelium appearance ranging from + (little) to +++ (very much); [2] levels of saccharified liquid appearance ranging from + (little) to +++ (very much); [3] number of gas discharges per 2 minutes; [4] standard deviation; [5] not detected; [6] values are means of triplicates; [7] means with different subscripts are statistically significant at the 95% confidence level.

of mixed-culture starter granules. In practice, rice and cassava flours are popularly used as ingredients in the production of rice wine starters in Vietnam. Although the practical reasons for using rice and cassava are known [6], no published knowledge is available about the effect of mixing ratio. If only rice flour is used, the starter tablet becomes too compact and hard so that the moulds can grow only on its surface. On the other hand, if only cassava flour is used, the starter tablet becomes too soft and spongy, which is assumed to limit the growth of yeasts.

3.2 The Feasibility of Pilot-scale Starter Processing

In line of preparation for the feasible application of new defined fungal starter processing into practice, in this experiment, the starter production was investigated at a larger amount (10 kg per batch) than those prepared at laboratory-scale processing, by using the selected conditions including rice flour 80% and cassava flour 20% with 4 days of incubation from the previous experiment. The amount of ingredients was divided into smaller parts in which each of 1 kg of ingredients was prepared on a stainless steel tray with a size of 39 × 39 × 9 cm. Three levels of inoculum including 10%, 20% and 30% w/w of ingredients were examined. The starter produced in the previous experiment was employed as the stock inoculation source. The performance of fungal starter granules in the saccharification and the alcoholic fermentation process from purple glutinous rice is presented in Table 2. The incubation temperature was set at 30 ℃; however, the regional ambient temperature was in a range at approx. 28-32 ℃ that was favourable for the growth of moulds and yeasts [8].

Following the same principle of the conversion in the saccharification and the alcoholic fermentation as mentioned in previous experiments, the mixed-culture starter granules produced with a larger amount as pilot-scale trial also gave the similar successful performance in such conversion way.

The treatment with the inoculation level of 10% gave the low performance in saccharification and fermentative abilities in comparison with other two treatments and the difference of alcohol contents was statistically significant at the 95% confidence level. However, the alcohol contents in the treatments with 20% and 30% of inoculation levels gave no significant difference. It was indicated that the level of mixed-culture starter of 20% favourable to be used as stock inoculum for the preparation of starter at larger scale processing.

3.3 Effect of Storage of Dry Defined Starter Granules on Winemaking Performance

Dry starter granules were vacuum packed and stored in polypropylene bags at ambient temperature (approx. 28-32 ℃). The objective was to examine the practical available way of storage and to minimize the influence of the limiting factors for the shelf-life and stability of starter, expectedly to apply easily and effectively into the actual conditions in practice. Dry matter contents and the winemaking performance of defined starter

Table 2 Winemaking performance in defined starter granules of different inoculation levels.

Level of inoculation (% w/w)	Mycelium appearance[1] (per day)			Saccharified liquid appearance[2] (per day)			Fermentation rate[3] at different time (h)				Alcohol	
	1	2	3	1	2	3	24	48	72	96	% v/v	SD[4]
10	+	++	+++	-[5]	+	++	12[6]	6	2	0	11 b[7]	1.7
20	+	++	+++	+	++	+++	14	8	1	1	14 a	1
30	+	++	+++	+	++	+++	18	9	2	1	14.33 a	1.15

[1] levels of mycelium appearance ranging from + (little) to +++ (very much); [2] levels of saccharified liquid appearance ranging from + (little) to +++ (very much); [3] number of gas discharges per 2 minutes; [4] standard deviation; [5] not detected; [6] values are means of triplicates; [7] means with different subscripts are statistically significant at the 95% confidence level.

Table 3 Effect of storage of dry defined starter granules on their moisture contents and performance in alcoholic fermentation.

Storage period (month)	Moisture content (%)	pH after fermentation	Alcohol	
			% v/v	SD[1]
0	7.8[2]	5	15.33 a[3]	0.57
2	8.5	5.1	13.33 b	1.15
4	9.59	5.2	13 bc	0
6	11.42	4.8	12 bc	2
8	11.38	5.2	11.33 c	0.57

[1] standard deviation; [2] values are means of triplicates; [3] means with different subscripts are statistically significant at the 95% confidence level.

granules before and after the storage are reported in Table 3.

During 8 months of storage at ambient temperature, the moisture levels of defined starter granules had gradually increased. This shows that the packaging may have no good moisture barrier function; however, the difficulty of protection of starter granules against such uncontrolled high ambient humidity and temperature levels is forecasted. It was found that after a certain period of storage, the higher moisture content the less performance of mixed-culture starter in the saccharification and alcoholic fermentation process. This finding is corresponding to the previous research report [7] in which the dry matter content is considerably noted as one of the factors influencing the shelf-life of the starter by effecting on the viability and activity of fungal cultures present in the starter.

Alternatively, the alcohol contents of the final product in winemaking somehow were used as the main factor indicating the assessment of the ability of mixed-culture starter during the storage. The results showed that the alcohol content significantly decreased in the treatment of 2 months of storage, as compared to the alcohol content of 15.3% v/v in the treatment before storage; however, there was no significant difference of alcohol contents in treatments during the storage periods from 2 months until 8 months of storage, in which a certain quite high level of alcohol was still obtained (11.3-13.3% v/v). This was obvious to indicate that, although the stability of mixed-culture starter granules had some challenge during the long storage of 8 months at ambient conditions, they could adapt somehow for their stable performance in winemaking. The pH values after the fermentation were in an identical range of 4.8-5.2 that should be supported for a normal successful alcoholic fermentation. Further trial will be needed into the storage stability of starter granules performed in local rice wine manufactures in order to have a proper recommendation of expiry use of starter granules when they are applied in practice.

3.4 Purification and Identification of Bacterial Contaminants

In the defined fungal starter processing, although only pure cultures of mould and yeast were used to inoculate the starter dough, the count of mesophilic bacteria was also determined to detect the presence of undesirable contaminants. A certain number of undesirable bacteria was found at approx. 2 Log CFU/g in the final defined fungal starter granules. On the other hand, although some contamination with undesirable bacteria occurred, mould and yeast still grew very well and dominated during the incubation as they performed highly their abilities in the alcoholic fermentation process. It was obvious these undesirable bacteria gave no competitive influences on the growth and the contribution of fungal cultures. However, although neither expect nor exclusively interested in mesophillic bacteria, it is necessary to characterize these bacterial isolates, particularly for the safety before making a transfer of the new product into practice, and this is the aim of this study.

Three bacterial isolates were obtained and named as BN06-31A, BN06-32 and BN06-33. They were characterized on the basis of their morphology, biochemical and physiological growth properties as well as the genetic partial 16S analysis. The results of the genetic analysis of three bacterial isolates were described in Fig. 1. The three bacterial isolates

belonged to the species named *Bacillus subtilis/amyloliquefaciens*. It meant that it was either *B. subtilis* or *B. amyloliquefaciens*. These two species of *B. subtilis* or *B. amyloliquefaciens*. were so much alike (100% identical) that was not possible to distinguish them with the genetic partial 16S results. Also, in the APICHB/20E test and the Biolog GP plate (STAG) test, the results could not differentiate these two species. There were no more tests to discriminate between *B. subtilis* and *B. amyloliquefaciens*. These bacteria might contaminate food but not cause food poisoning and they are not considered as a human pathogen. Particularly, some species of *Bacillus* can be commercially employed for the production of fermented foods, such as *Bacillus natto* in *natto* processing (Japan), *cheonggukjang* (Korea).

3.5 Assessment of Defined Starter Granules in the Winemaking Fermented from Different Agricultural Starchy Materials

In the context of the aim to pave the way for applying the experimental superior fungal starter into practice, in this experiment, the ability of defined starter was tested in the winemaking from different available starchy materials in the region and its validation in comparison with commercial starters were assessed in the winemaking.

Three kinds of starters including defined mixed-culture starter, Hai Anh Quang starter and Phuc Hung starter and four different starchy materials including purple glutinous rice, Phu Tan glutinous rice, Huong Lai rice and Huyet Rong rice were assessed and compared. The results of winemaking performance in defined starter granules and commercial starters of four different starchy materials were presented in Table 4.

During the saccharification stage and the alcoholic fermentation process, the results showed that, in generally, all testing starters similarly performed followed the normal principle in the winemaking from all different kinds of starchy resources. During the incubation in the saccharification process the mycelium appearance and the liquefaction were increasingly obvious, as well as the total high saccharified sugar levels (21-32 °Brix) were obtained. In the alcoholic fermentation stage, the rapidly high fermentation rate was observed after 1 day of incubation, then increased after 2 days and almost no more fermentation signal was observed after 4 days in all treatments.

By assessing the alcoholic contents of the final wine as the main factor indicating the starter ability, the results showed that in the treatments of commercial starter inoculation the alcohol contents differently varied depending on the kinds of starchy materials

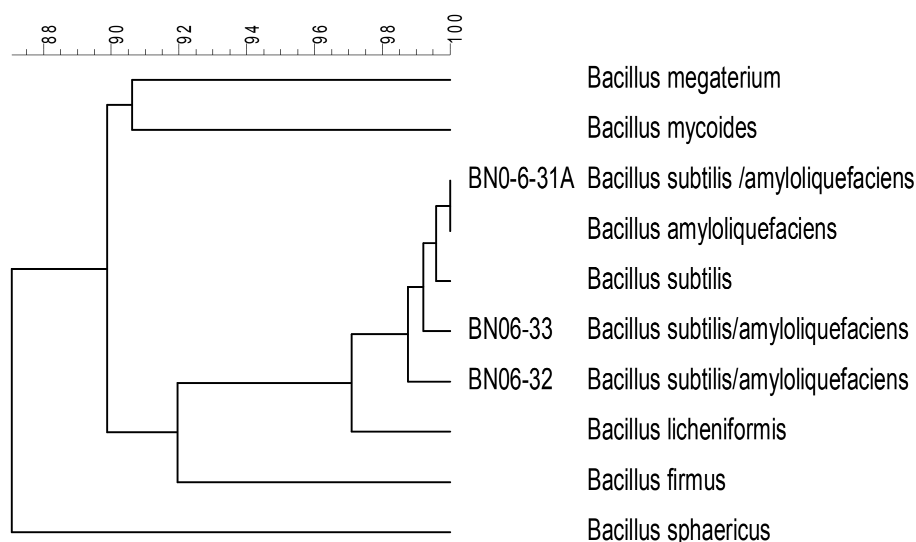

Fig. 1 The genetic analysis of bacterial isolates BN06-31A, BN06-32 and BN06-33.

Table 4 Winemaking performance in defined starter granules and commercial starters of different starchy materials.

Starters	Starchy materials	Saccharified liquid appearance in mould fermentation (per day)[1]			Fermentation rate[2] at different time (h)				Alcohol	
		1	2	3	24	48	72	96	% v/v	SD[3]
D[4]	PGR[5]	+	++	+++	35[6]	14	2	1	14.33 ab[7]	0.57
HAQ[8]	PGR	+	++	+++	28	12	1	0	11 d	0
PH[9]	PGR	+	++	++	24	14	2	1	10.67 d	0.57
D	PT[10]	-[11]	+	++	25	15	4	3	13.33 bc	1.52
HAQ	PT	+	++	++	21	13	2	0	13.67 bc	0.57
PH	PT	+	++	+++	34	10	10	4	14.33 ab	0.57
D	HL[12]	+	++	+++	35	16	2	2	15 a	0
HAQ	HL	+	+	++	26	15	2	2	13.67 bc	0.57
PH	HL	+	+	++	27	14	2	1	13.33 bc	0.57
D	HR[13]	-	+	++	32	13	2	1	14.33 ab	0.57
HAQ	HR	-	+	+	30	12	1	1	12.67 c	0.57
PH	HR	-	+	+	24	13	2	2	10.67 d	1.52

[1] levels of saccharified liquid appearance ranging from + (little) to +++ (very much); [2] number of gas discharges per 2 minutes; [3] standard deviation; [4] defined fungal starter; [5] purple glutinous rice; [6] values are means of triplicates; [7] means with different subscripts are statistically significant at the 95% confidence level; [8] Hai Anh Quang starter; [9] Phuc Hung starter; [10] Phu Tan glutinous rice; [11] not detected; [12] Huong Lai rice; [13] Huyet Rong rice.

employed, whereas the high levels of alcohol contents were stably achieved in all treatments of defined starter performance. This can indicate that the experimental mixed-culture fungal starter granules could be able to be employed effectively as the inoculum for the rice wine fermentation from different kinds of available agricultural starchy materials in the region. The defined starter also received the highest performance for its function during the alcoholic fermentation process, as compared to this of other commercial starters.

4. Conclusion

The mould of *Amylomyces rouxii* (CBS 111757; LU 2043) and the yeast of *Saccharomyces cerevisiae* (LU 1250) were successfully applied as the inoculation source for the defined starter processing. As a logical continuation of previous findings with the ultimate aim for the application of experimental defined starter processing into practice, a pilot-scale testing trial of the starter preparation was prepared following the selected favourable conditions of mixed ingredients, incubation time and inoculum level. During 8 months of storage at ambient temperature (approx. 28-32 ℃) dry starter granules could adapt for their stable performance in winemaking. As the undesirable bacteria, Bacillus subtilis/amyloliquefaciens were found at small amount and gave no competitive effect to the performance of mixed-culture fungal starter granules. The defined starter also performed effectively in winemaking from different agricultural starchy resources. However, in spite of the successful ability for winemaking in testing trials, the defined starter granules should also performed well in the actual conditions of rice wine production at local manufactures. It will therefore be necessary to supply a stock inoculum of defined starter granules to lead local manufactures for further testing in the rice wine fermentation. This aspect is currently examined in another separate research.

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Studies on Feeding Habits and Parasitological Status of Red Fox, Golden Jackal, Wild Cat and Stone Marten in Sredna Gora, Bulgaria

Zvezdelina Kirkova[1], Evgeniy Raychev[2] and Dimitrina Georgieva[1]

1. Department of Parasitology, Trakia University, Stara Zagora 6000, Bulgaria

2. Department of Game Animals, Trakia University, Stara Zagora 6000, Bulgaria

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Abstract: In order to define the role of wild carnivores in the epidemiology of parasitoses with veterinary and medical importance their parasitological status and feeding habits were studied. In the period 2001-2006 the feeding habits of 167 foxes, 78 jackals, 40 wild cats and 23 stone martens from the area of Sredna Gora, Bulgaria were investigated. 113 of the foxes, 56 of the jackals, 22 of the wild cats and 21 of the martens were subjected to helminthological study. 147 wild boars and 26 badgers from the same area were subjected to trichinelloscopy. Rodents were the main food of the wild cats (82.7%), martens (52%) and foxes (50%). The main food of the jackals was carrion from domestic and wild animals (79.5%). 95.5% of the foxes, 100% of the jackals, 95.5% of the wild cats and 89% of the stone martens were infected with one or more helminth species. The prevalence of the most important helminths: *Trichinella* spp., *Taenia* spp. and *Ancylostoma* spp. was high in all carnivores examined. In the infected with *Trichinella* spp. animals only *T. britovi* was demonstrated. The wild boars and badgers were not infected with *Trichinella* spp. The correlation between the feeding habits and parasitological status is discussed.

Key words: Feeding habits, wild carnivores, parasitological status, helminthes, *Trichinella britovi.*

1. Introduction

Previous studies in Bulgaria show that wild carnivores are hosts for a range of parasites [1-4]. Their results show differences in the parasitic fauna of wild carnivores from different areas. Infected wild carnivores move about freely and become the main source of animal and human infection.

According to ecological and epidemiological data foxes helminth fauna is related mainly to their diet and less to the sex and age [4-6]. On the other hand wild carnivore's diet depends on variation in their prey population [7].

The number of foxes and stone martens in Sredna gora is stable, whereas that of jackals has considerably increased during the last 20 years [8]. Expansion of jackals is observed [9]. The number of wild cat population changes considerably because of frequent conflagrations. In the area wild carnivores cohabit with stray dogs and cats that inhabit urban areas and probably transmit infections between natural and synantropic foci.

The aim of the study was to investigate the feeding habits and parasitological status of the most common carnivores and their role in the epidemiology of parasitoses with veterinary and medical importance.

2. Materials and Methods

2.1 Study Area

The study area is situated in South Bulgaria, The

Corresponding author: Zvezdelina Kirkova, M.Sc., head assistant professor, research fields: veterinary parasitology, helminthology. E-mail: z.t.kirkova@abv.bg.

State Forestry in Stara Zagora (φ = +42˚25′, λ = 25˚37′) (Fig. 1).

These are 40,936 ha low mountains. These are hills (altitude from 350-400 m to 800-900 m) with dry and warm climate. The area is transitional to Mediterranean precipitation regime.

The main deciduous trees *Qercus pubescens*, *Q. cerris* and *Q. frainetto* are mixed with *Carpinus orientalis*. There are some artificially planted coniferous forests (*Prunus nigra*). Bushes like *Crataegus monogyna*, *Rosa canina*, *Rosa gallica*, *Prunus spinosa*, *Ligustrum vulgare*, *Cornus mas* are common for the area.

The deciduous forests and bushes are mixed with uncultivated lands, abandoned lets, after agricultural activity and abandoned fruit gardens. A lot of cutting and hunting activity takes place in the area.

Other terrestrial predators present in the study area comprise *Mustela putorius*, *Mustela nivalis*, *Meles meles* and rarely *Canis lupus*.

The most important prey species are large ungulates: *Cervus elaphus*, *Capreolus capreolus*, *Sus scrofa*, sometimes *Dama dama* and rodents, mainly: *Apodemus agrarius*, *Sylvaemus flavicollis*, *Mus musculus*, *Microtus arvalis*, *Arvicola terrestris*, *Ratus norvegicus* and *Rattus rattus*.

Birds as a potential prey are: *Scolopax rusticola*, *Columba palumbus*, *Perdix perdix*, *Alectoris chucar*, *Phasianus colchicus* and *Streptopelia decaocto* in suburban areas. Typically forest birds inhabit the area: *Garrulus glandarius*, *Turdus merula* and *Picidae* spp..

Fig. 1 Study area.

The objects of our study coexist with a lot of stray dogs and cats. In winter time the dogs move together in groups and hunt like wolves. Urban areas - villages are situated at 5-10 km distance from each other.

2.2 Necropsy and Parasitological Examinations

During 6 autumn and winter seasons (2001-2006) a total of 167 foxes, 78 jackals, 40 wild cats and 23 stone martens were collected by different ways of shooting. The carcasses were obtained from gamekeepers and hunters. Stomachs were removed from carcasses; the content was taken out and investigated in the lab of Trakia University. Constituents were identified by comparison with a reference collection of potential pray items and from the information given in guides [10-12]. Some of the constituents were identified to species level. Sometimes the identification of the rodents found in foxes and wild cats stomachs was possible because they were swallowed whole or cut in two parts. The contribution of each kind of food to the diet was presented as a percentage of occurrences in the samples. Suspected parasite tumors were removed from the wall of the stomach and dissected. The material for trichinelloscopy was taken from *crura diafragmatica*. Twenty-four pieces were obtained from each animal. Isolates were obtained from 15 foxes, 10 jackals, 6 stone martens and 5 wild cats naturally infected with *Trichinella* spp.. These isolates were subjected to speciation by both multiplex PCR and cross-breeding experiments in the Institute of Parasitology (Bern, Switzerland).

113 of the foxes, 56 of the jackals, 22 of the wild cats and 21 of the martens were subjected to helminthological study. The carcasses were frozen at –20 ℃ for minimum 10 days and therefore left overnight to defrost before examination. The organs of gastrointestinal tracts were removed and placed in separate containers. Small intestines were cut open along their length in a container with water and the mucosa was scraped. The material was examined in

portions in Petri dishes on a dark background. Trematodes and cestodes were collected in water, nematodes and acanthocephales in saline. The large intestines were open along their length and all visible parasites removed. Nematodes and acanthocephales were examined microscopically. Cestodes were stained in HCL-or lactic acid-soluble carmine and mounted in Canadian balsam. Helminths were identified by means of guides [3, 13-15]. Because carcasses were frozen for several days the identification of the most *Mesocestoides* spp. and *Taenia* spp. was not possible.

The rectal contents were examined for parasite eggs and oocysts using the Fulleborn flotation technique. Kidneys were cut up and pelves inspected. Bladder were opened in Petri dish and examined on a dark background. The heart, pulmonary artery and lungs were opened and inspected for presence of *Dirofilaria immitis*. The specimens of the lungs were cut into pieces in a conical glass with warm 37 ℃ water. After an hour the sediment was examined microscopically. The bile was collected in conical glasses and the sediment was examined microscopically.

Meat samples from 147 wild boars and 26 badgers from the same area were subjected to trichinelloscopy.

Specimens of the parasites found were deposited in the Parasitological Museum at the Department of Parasitology, Veterinary Faculty, Trakia University, Bulgaria.

3. Results and Discussion

3.1 Autumn and Winter Food of Examined Predators

The diets of four studied species are shown in Table 1.

Rodents are the bulk of the fox diet (50% of full stomachs). The second place (35.5%) takes remains of wild animals and 21.2% - from domestic animals as carrion. It can be assumed that the large mammals - red deers, roe deers, wild boars and stock animals, foxes consume as carrion. Deer's or wild boar's skins can be often found in the forest, left from hunters or poachers.

The identification of the species *Sylvaemus sylvaticus*, *S. flavicollis, Microtus arvalis* and *Rattus norvegicus* was possible because of the fact that the fox swallow most of the small rodents whole. *Lepus capensis* is present only in 4% of occurrence. The percentage of birds in the fox stomachs is only 18.8%. Feathers from *Turdus merula, Garrulus glandarius, Perdix perdix* and *Paridae spp*. were recognized. The plant remains consists mainly of *Rosa canina* berries.

Rodents were found to be only 15% of the full jackal's stomachs. The main food for this bigger predator was carrion -79.5% of occurrence. The correlation between wild and domestic animals was approximately 1:1. Hares occupied only a minor position (1.6%) on the diet of the jackals.

The main food sources for the stone marten in the autumn and winter seasons were rodents (52%) of occurrence. Game animals (30.4%) and insects in dormancy (21.7%) occupied the second place of occurrence. Carrion from domestic stock (13.05%) and plants (8.69%) were less important as a potential prey for the stone martens.

The rodents constitute an easy source of food for the wild cat (82.5% of the stomachs). Wild birds take the second place (12.5%). Most of the rodents were determined because they were swallowed whole or cut in two parts. *Microtus arvalis* and mainly *Sylvaemus sylvaticus* were identified. The birds recognized were: *Passeriformes* spp.*, Paridae* spp.*, Phasianus colchicus* and *Perdix perdix*. It could be seen from Table 1 that wild cat very rarely consume in the autumn and winter seasons reptiles, carrion from sheep, roe deer and fox.

3.2 Parasitological Status

No parasites were found in 4.4% of the foxes examined.

In foxes parasitism involving only 1 species was found in 9.3% of the cases, 2 species in 24.2%, 3 species in 17.4%, 4 species in 28.3%, 5 species in 6.6%, 6 species in 5.4% and 7 species in 4.4%. 100% of the jackals examined were infected: 18.5% with 1

Table 1 Food of foxes (*Vulpes vulpes*), jackals (*Canis aureus*), wild cats (*Felis silvestris*) and martens (*Martes foina*) in the autumn and winter seasons.

Food ingredients	*Vulpes vulpes* (n = 167)	*Canis aureus* (n = 78)	*Felis silvestris* (n = 40)	*Martes foina* (n = 23)
1. Rodents	50	15.0	82.5	52.17
2. Domestic animals:	21.2	39.7	5.0	13.05
- Rabbit *(Oryctolagus cuniculus)*	1.6	1.6	-	-
- Cat (*Felis catus)*	1.6	5.0	-	4.35
- Pig (*Sus scrofa domestica)*	4.0	6.6	-	4.35
- Sheep (*Ovis aries)*	1.6	8.3	2.5	4.35
- Dog (*Canis familiaris)*	3.3	10.0	-	-
- Hen (*Gallus domesticus)*	12.3	6.6	2.5	-
- Horse (*Equs caballus)*	-	1.6	-	-
3. Game animals:	35.9	39.8	27.5	30.43
- Wild birds	6.5	5.0	12.5	4.35
- Wild boar (*Sus scrofa*)	12.3	11.6	-	-
- Roe deer (*C. capreolus*)	7.4	4.2	2.5	13.04
- Fallow deer (*Cervus dama*)	1.6	-	-	-
- Red deer (*Cervus elaphus*)	0.8	4.2	-	-
- Fox, Jackal, Badger	3.3	13.3	2.5	-
- Hare (*Lepus capensis*)	4.0	1.6	10.0	13.04
4. Insects	0.8	-	-	21.74
5. Fish	0.8	3.3	-	-
6. Earthworms	3.3	-	2.5	-
7. Amphibians	1.6	0.8	-	-
8. Reptiles	0.8	1.6	2.5	4.35
9. Plants	11.5	16.6	-	8.69
10. Food scraps	3.3	10.0	-	-
Unidentified	-	1.6	-	-
Full stomachs	122	60	40	23

species, 28.9% with 2 species, 31.5% with 3 species, 18.5% with 4 species and 2.6% with 8 species.

The parasites found and their prevalence among the total fox and jackal sample is shown in Table 2.

The only one trematoda species found *Alaria alata* is rare in foxes (4.1%) and jackals (1.9%). The most prevalent cestods in foxes and jackals are *Mesocestoides* spp. (73.4% and 34.6% respectively) following by *Taenia* spp. (15.3% and 23%) and *D. caninum* (1% and 3.8%). Because of freezing the most specimens of *Mesocestoides* spp. were disintegrated. Hooks were missing from the most Taeniids making their identification at the species level impossible. The following *Taenia* species were identified: 7 specimens *T. polyacantha* obtained from foxes, 4 specimens *T. hydatigena* and 2 specimens *T. pisiformis* obtained from jackals. Both *M. lineatus* and *M. litteratus* were found in foxes and jackals. *E. granulosus* was found only in jackals (1.9%).

A total of 10 nematoda species were recovered from the foxes. The most common were *C. plica* (56.7%), *U. stenocephala* (55.1%) and *Trichinella* spp. (29.5%).

Nine nematoda species were found in jackals. The most prevalent were *U. stenocephala* (84%), *Trichinella* spp. (40%) and *T. vulpis* (30.7%).

One acanthocephala species *M. catulinus* was found in foxes and jackals (3.1% and 3.8% respectively).

No parasites were found only in 1 (4.5%) of the wild

Table 2 Prevalence of helminth and protozoa parasites among foxes (n = 113) and jackals (n = 56).

Species	Prevalence (%)	
	Foxes	Jackals
Trematoda		
Alaria alata	4.1	1.9
Cestoda		
Echinococcus granulosus	0	1.9
Taenia sp.	15.3	23
Dipylidium caninum	1	3.8
Mesocestoides sp.	73.4	34.6
Nematoda		
Ancylostoma caninum	0	11.5
Uncinaria stenocephala	55.1	84.6
Toxocara canis	21.4	7.7
Toxascaris leonina	6.1	5.8
Trichinella sp.	29.5	40
Trichuris vulpis	12.2	30.7
Capillaria plica	56.7	16.4
Capillaria aerophyla	2	0
Spirocerca lupi	24.6	0
Rictularia affinis	15.3	7.7
Dirofilaria immitis	3.1	9.6
Acanthocephala		
Macracanthorhynchus catulinus	3.1	3.8
Protozoa		
Sarcocystis sp.	2	1.9
Isospora sp.	4.1	5.8
Eimeria sp.	5.1	5.8

Table 3 Prevalence of helminth and protozoa parasites in wild cats (n = 22).

Species	Prevalence (%)
Cestoda	
Taenia taeniaeformis	90.9
Mesocestoides sp.	36.4
Nematoda	
Ancylostoma tubaeforme	68.2
Toxocara mystax	81.8
Capillaria plica	31.8
Capillaria aerophyla	4.5
Trichinella sp.	45.5
Spirocerca lupi	27.3
Physaloptera praeputialis	31.8
Protozoa	
Isospora sp.	9.1
Eimeria sp.	9.1

cats examined (Table 3). 2 species were found in 9.1%, 3 species in 22.7%, 4 species in 18.2%, 5 species in 9.1%, 6 species in 18.2% and 7 species in 18.2%.

Most prevalent nematods were *T. mystax* (81.8%), *A. tubaeforme* (68.2%) and *Trichinella* spp. (45.5%). 18 of the 22 wild cats examined were infected with both *T. mystax* and *T. taeniaeformis*.

About 89% of martens harboured parasites (Table 4). 33.3% harboured 1 species, 33.3% - 2 species and 22.2% - 4 species.

The most prevalent were *Toxocara* spp. (54.5%), *Capillaria plica* (44.4%) *and Trichinella* spp. (28.6%).

In the infected with *Trichinella* spp. foxes, jackals, stone martens and wild cats only *T. britovi* was demonstrated. Results obtained by molecular typing fully matched those of cross-breeding [16].

Coccidian oocysts (*Isospora, Sarcocystis* and *Eimeria*) were found in a low proportion of foxes, jackals, wild cats and martens.

The trichinelloscopy of the meat samples obtained from wild boars and badgers gave negative results.

The results of the study show that foxes, jackals, wild cats and martens from Sredna gora are infected with a range of helminth and protozoa parasites. Predominant are biohelminths whose intermediate and additional hosts are rodents. The previous studies in Bulgaria showed similar results. In Northwestern Bulgaria, 95% of the foxes are infected with 1 to 10 helminths species [4]. In one young fox from South-west Bulgaria are found 12 helminth species [3]. According to authors in winter and autumn the foxes are infected with a larger range of helminth species

Table 4 Prevalence of helminth and protozoa parasites in martens (n = 21).

Species	Prevalence (%)
Cestoda	
Taenia sp.	9.1
Hydatigera taeniaeformis	18.2
Nematoda	
Ancylostoma sp.	9.1
Toxocara sp.	54.5
Capillaria aerophyla	11.1
Capillaria plica	44.4
Trichinella sp.	28.6
Protozoa	
Isospora sp.	11.1

than in spring and summer [3, 4]. High extensity of infection with one or more gastrointestinal helminths is reported for foxes [6, 17, 18].

The trematoda species *A. alata* which intermediate hosts are amphibians is also found in 19.4% of the foxes from South-west Bulgaria and in 2.1% of the foxes from North-west Bulgaria [3, 4].

The prevalence of *Mesocestoides* spp. in foxes is considerably higher than in jackals. That may be explained with the higher percentage of rodents in fox's diet (50%) than in jackal's diet (15%). The results of the study and those of previous studies show that the foxes in Bulgaria are with high extensity and intensity of invasion with *Mesocestoides* spp. [1, 3, 4].

With the majority of rodents could be explained the high prevalence of *T. taeniaeformis* in wild cats. Similar results were reported for wild cats from Germany [19]. *T. taeniaeformis* is found in one of the 243 foxes examined [4]. The negative result confirms their opinion that foxes are nonspecific host for *H. taeniaeformis*.

The prevalence of *E. granulosus* in jackals (1.9%) is considerably lower than those reported for jackals (30.7%) from the same area [20]. The negative results in foxes sustain the assertion that foxes do not play a role in the epidemiology of the echinoccosis [21]. Whereas *E. granulosus* is found in 0.2% and 0.1% of the foxes in Iberian Peninsula and South England respectively [5, 6].

The prevalence of *Trichinella* spp. is high in all carnivores examined which correlate with consumption of rodents. A noticeably higher prevalence of *Trichinella* spp. (45.5%) in wild cats than in foxes (29.5%) and martens (28.6%) could be related to the higher consumption of rodents. In jackals the prevalence is high in spite of the lower consumption of rodents. But in their diet they include high percentage carrion of boars and predators (foxes, jackals, badgers, wild cats and dogs). Considerably higher prevalence of *Trichinella* spp. in martens (61.9%) from the same area is detected previously [2].

The high prevalence of *C. plica* in foxes, jackals, wild cats and martens -56.7%, 16.4%, 31.8% and 44.4% respectively, is related to the consumption of earthworms. Earthworms comprise a small proportion of the studied carnivore's autumn and winter diet, but in the summer earthworms are present in 13% of fox's stomachs, more than in jackals 3% [8].

Spirocerca lupi was found in foxes (24.6%) and wild cats (27.3%). Although insects constitute a significant proportion of the marten's summer diet and low proportion of the jackal's diet, *S. lupi* was not found in martens and jackals.

D. immitis is more prevalent in jackals (9.6%) than in foxes (3.1%). According to previous studies the prevalence of *D. immitis* in Bulgaria is 7.4% in dogs, 5.2% in foxes, 4.4% in jackals and 5.5 % in wolves [22]. *D. immitis* is found in 11% of the foxes in Spain [23].

Dioctophyme renale was not found in the carnivores examined. This species is detected in martens from Ontario, Canada [24]. In Bulgaria there are two reports for human dioctophymosis and one of the cases is in a man from Stara Zagora [25]. Previous studies on fox's helminth fauna show the lack of heminths whose intermediate hosts are fishes. The lack of *D. renale* could be explained with the low percentage of fish in the carnivore's diet. Fish was found in the stomachs of one fox and one jackal.

The prevalence of *M. catulinus* in foxes and jackals (3.1% and 3.8% respectively) is similar to previously observed for foxes from the same area [1].

In autumn and winter the birds, reptiles, amphibians and fishes comprise a small proportion of the studied carnivore's diet. It seems reasonable to assume that their role for infection of wild carnivores is insignificant.

In jackals the prevalence of the geohelminths *U. stenocephala* (84.6%) and *T. vulpis* (30%) is considerably higher than in foxes (55.1% and 12.2% respectively). It seems clear that consuming carrion jackals swallow contaminated soil. Litter, sticks, soil and animal faeces were often found in the jackal's stomachs. The previous investigations show that in Bulgaria *U. stenocephala* and *T. vulpis* are the most

common gastro-intestinal helminths in dogs [26]. Attempts to control these helminths in dog populations might, therefore, be hindered by the presence of the same helminth species in wild carnivores.

4. Conclusions

The results of the study are in maintenance of the opinion that wild carnivores are reservoir and source of animal and human infection with trichinellosis, larval cestodoses and other helminths. The prevalence of some helminths in wild carnivores is closely related to their feeding habits and host specificity. Rodents are the main source of infection of foxes, wild cats and stone martens with helminths, whereas, for jackals the carrion is the main source of infection.

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Seasonal Variation in Krasnodar Greenbug Population for Virulence to Sorghum Genotypes

Eugene Radchenko and Anton Zubov

Department of Genetics, Vavilov Institute of Plant Industry, St.-Petersburg 196600, Russia

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Abstract: Intraspecific variability in *Schizaphis graminum* (Rondani) was studied in 2002-2005. Aphid clones were sampled from sorghum field at the Kuban Experimental Station (Krasnodar region, Northern Caucasus, Russia) in June (active greenbug migration to the field), July (high rate of the population increasing), and August (greenbug abundance decreasing) and at the end of September or in the beginning of October (appearance of sexual males and females). Damage rating was estimated for two plant sets composed of three sorghum differentials. Set A contains Deer (gene for resistance *Sgr*4), Sarvasi (*Sgr*1 + *Sgr*2) and Capbam (*Sgr*12). Set B contains Shallu (*Sgr*3), Sorgogradskoe (*Sgr*5) and Durra Belaya (*Sgr*5 + *Sgr*6). To estimate variability in greenbug subpopulations criteria proposed by Zhivotovsky (1982) were used. Frequencies of greenbug clones with virulence to sorghum accessions essentially differed. Very high overall and seasonal polymorphism of the insect for virulence was revealed. Among 517 aphid clones tested 33 phenotypes for virulence were identified. The aphid subpopulations collected from the same field at different periods of sorghum vegetation varied significantly in share of rare phenotypes. Criteria of similarities varied from 0.268 to 0.739; according to criterion of identity significant differences between 44 summer subpopulations from 66 studied were found.

Key words: Sorghum genotype, greenbug, virulence, seasonal variation.

1. Introduction

One of the main factors affecting the stability of cereal resistance to aphids is the appearance of insect biotypes with new virulence that are capable to damage previously resistant varieties. Distinctions between populations of greenbug, *Schizaphis graminum* (Rondani), with respect to ability to feed on definite varieties of cereal crops, were discovered for the first time in the United States in 1947 [1]. At present, no less than ten bio-types [2] are known for greenbug, and five biotypes are known for corn leaf aphid *Rhopalosiphum maidis* (Fitch) [3]. Differential interaction with host plants was shown for Russian wheat aphid *Diuraphis noxia* (Mordvilko) [4], English grain aphid *Sitobion avenae* (Fabricius) [5] and bird cherry-oat aphid *Rhopalosiphum padi* (L.) [6]. The possibility of adaptation to host necessitates the study of insect population variations the causes of which can be immigration of virulent clones to local population, mutations and combinative variability for genes for virulence in local populations.

The nonuniformity of *S. graminum* populations in the countries of former USSR was revealed for the first time in the study of the resistance of two sorghum entries to the Stavropol and Uzbek aphid populations [7]. Further experiments confirmed the existence of differences in damage ratings of entry k-1362 and variety Sarvasi after infestation with eight European and two Asian populations of the phytophage. The resistance genes in sorghums k-1362 and k-9436 are effective only against European greenbug populations and while cv. Sarvasi is only effective against Asian populations. Within the Krasnodar population there are at least two biotypes, differing in their virulence to Sarvasi [8].

Corresponding author: Eugene Radchenko, Ph.D., research field: plant resistance. E-mail: eugene_radchenko@ rambler.ru.

From 1993-2001, the frequency of virulent phenotypes (biotypes) in the Krasnodar population of *S. graminum* was estimated using five sorghum differentials with resistance genes *Sgr*1 – *Sgr*6. The total 12 aphid phenotypes were identified. The most common phenotype heavily damaged cultivar Sarvasi (*Sgr*1 + *Sgr*2) and entries with resistance genes *Sgr*3, *Sgr*4. The structure of the aphid population is in terms of the frequency of certain phenotypes and share rare phenotypes differed between years [9, 10]. The significant difference between the Krasnodar and Saratov greenbug populations in the frequency of virulent phenotypes in 1999 indicates that it is possible for populations to exist in isolation in the European part of Russia. The European populations of this insect are relatively isolated from the Asian populations (Uzbekistan, Kazakhstan). The genes for resistance in cultivar Sorgogradskoe k-9436 (*Sgr*5) and entry k-1362 (*Sgr*5 + *Sgr*6) are effective only against the European populations of the aphid, and that of cultivar Sarvasi against those in Asia. It is likely that greenbug biotypes capable of damaging resistant cultivars are present before commercial cultivation of these cultivars and that genetic uniformity promotes distribution of aphid clones with specific virulence [9, 10].

It must be mentioned that almost all modern conclusions on the genetic structure of aphids and other harmful organisms' populations are based on analyses of samples collected in certain regions only one time. This methodological approach can obviously result in misconception because of possible inter- and intra-seasonal changes in frequencies of virulence genotypes. Ignoring this point can in some extent lower the value of existing concepts on genetic structure of the aphid. Moreover, several aspects of aphid microevolution, seasonal dynamics of the genetic structure, and existing of genetically different populations on different genotypes will not be known.

The general aim of the work was to study the range of seasonal variation of virulence phenotypes of a greenbug population in Krasnodar region.

2. Materials and Methods

2.1 Insects

Intraspecific variability in *S. graminum* was studied from 2002-2005. Aphids were sampled on heavily damaged sorghum plants from a single field at the Kuban Experimental Station of Vavilov Institute of Plant Industry (Krasnodar region, Northern Caucasus, Russia) in June (active greenbug migration to the field), July (high rate of the population increasing), August (greenbug abundance decreasing) and at the end of September or in the beginning of October (appearance of sexual males and females). In 2004, we had only 3 collections (June-August). At all 15 aphid collections were sampled in 4 years. For transportation of aphids, we used glass containers with wheat seedlings, where 1 colony of the greenbug was maintained. To obtain greenbug clones under laboratory conditions in St.-Petersburg, several sprouting seeds of wheat variety Leningradka were placed on the cotton wool wetted with water in bottom of Petri dish. In 3-5 days, one female was then transferred to a seedling in each dish and isolated by glasses; the top was covered with cloth. Cages with clones of the aphid were maintained in the light chamber at 21 ± 1 ℃ and 16 : 8 h photoperiod under "cool-white" fluorescent lighting. The further maintenance of clones was carried out by shaking off insects into similar cages [11]. Overall 517 clones were analyzed varying 5-58 for each sample.

2.2 Plants

Damage caused by *S. graminum* was estimated using 6 sorghum entries (cultivars and germplasms). Sarvasi (k-3852, Hungary) had dominant (*Sgr*1) and recessive (*Sgr*2) genes for greenbug resistance, Shallu (k-9921, USA) carried incompletely dominant resistance gene *Sgr*3, Deer (k-6694, USA) was protected by the dominant gene *Sgr*4 and Capbam (k-455, USA) had the dominant gene *Sgr*12. These genes expressing resistance were only against certain clones of *S. graminum*. Sorgogradskoe (k-9436, Rostov region)

and entry Durra Belaya (k-1362, Syria) had a semidominant gene (*Sgr*5) for resistance against the Krasnodar aphid population, and k-1362 additionally the recessive gene *Sgr*6 [12, 13].

2.3 Plant Resistance Evaluation

Seeds of differentials and the susceptible control (Nizkorosloe 81) were sown in soil in pots arranged in a circle, isolated by glass cages, and plants at the seedling stage were infested with 517 clones of the aphid. When the control plants died, damage in the other plants was estimated according to the scale [14]: 0: no damage; 1: 1-10%; 2: 11-20%; 10: 90-100% of the leaf surface damaged. There were 2 sets of the experiment with 3 resistant entries and a susceptible control. Damage ratings 1- 4 were referred to resistance (R) and that 9-10 to susceptibility (S).

2.4 Data Analysis

Population polymorphism was estimated as the frequency of phenotypes, identified using the above entries. A difference in the pest phenotypes was indicated when the damage rating by a given aphid clone significantly differed from that for other clone by one plant entry.

To designate clone phenotypes, a coding system was used. Sorghum entries were divided into two plant sets composed of three differentials in a certain order. Set A contained Deer, Sarvasi, Capbam; and set B contained Shallu, Sorgogradskoe, Durra Belaya. Host set reactions were coded from 0 to 7. In the case of clone avirulence (resistance of the plant entry = R) index 0 was assigned. In the case of virulence (susceptibility of the plant entry = S) indexes 1, 2, and 4 were assigned to 1st, 2nd and 3rd samples, respectively (2^{n-1}, where n: the number of the sorghum entry in the group). For each clone, the index for the group was calculated as a sum of indices for each entry. Index for the clone for all entries was comprised of two consecutive indices from each set [15].

The variability of a greenbug population was estimated using the criteria proposed by Zhivotovsky [16]. Intrapopulation variability was estimated with the help of criterion μ (average phenotype number in a population) according to the formula:

$$\mu = (\sqrt{p_1} + \sqrt{p_2} + ... + \sqrt{p_m})^2 \qquad (1)$$

where p_1, p_2..., p_m: frequencies of phenotypes; m: phenotypes number. Error S_m was calculated according to the formula:

$$S_m \approx \sqrt{\frac{\mu - (m-\mu)}{N}} \qquad (2)$$

where N: number of clones.

In addition to average phenotypes number, the parameter h: share of rare phenotypes was calculated:

h = 1 - μ /m, and also error:

$$s_h \approx \sqrt{\frac{\mu - (m-\mu)}{N}} \qquad (3)$$

If μ gives an estimation of a degree of population variability, the parameter h estimates structure of this variability.

To compare subpopulations under study we used criterion of similarity r:

$$r = \sqrt{p_1 q_1} + \sqrt{p_2 q_2} + ... + \sqrt{p_m q_m} \qquad (4)$$

where p_i and q_i are frequencies of phenotypes per compared dates of greenbug samples.

Significance of differences between populations for frequencies of common phenotypes was estimated according to criterion of identity I:

$$I = \frac{8N_1N_2}{N_1+N_2}(1 - r - \frac{p^0+q^0}{4}) \qquad (5)$$

where p^0: sum of phenotypes frequencies in 1st sample which are not represented in 2nd sample; q^0: sum of phenotypes frequencies in 2nd sample, which are absent in 1st one.

3. Results

3.1 Population Polymorphism

The highest aphid infestation at sorghum collection site, and consequently plant damage was recorded in 2002. The damage of standard cv. Kubanskoe krasnoe 1677 sown after each 20 sorghum collection entries

was 5-7 scorings. Environmental conditions (high temperature, absence of cloudbursts, and low number of aphidophage) appeared to favor an outbreak of the aphid at the end of July. In following years, aphid numbers were low and the damage Kubanskoe krasnoe 1677 did not exceed scoring 3.

The largest summer samples of clones (subpopulations) were analyzed in 2002 (Table 1). In fall collections amphygonic generation (sexual males and females) dominated during 2 years resulted in only small quantity of material analyzed. At the end of September in 2002, only 5 aphid clones of 24 were parthenogenetic ones and 19 were amphygonic. However in September of 2005 all 17 clones collected in the field reproduced parthenogenetically.

Damage to Durra Belaya and Deer caused by avirulent clones did not exceed a score of 2. A rather wide variation in damage was characteristic for Sarvasi (scores 1-3), Capbam and Sorgogradskoe (scores 2-4). Damage to Shallu caused by avirulent clones was mainly an index 3.

Significant seasonal variation of frequencies of *S. graminum* clones virulent to 5 entries was revealed (Figs. 1 and 2). Only clones heavily damaging Durra Belaya were identified with invariably low frequency (from 0.6% till 5.4% each year). In 4 years of investigation share of clones heavily damaging Sorgogradskoe and Shallu increased distinctly.

Among 517 clones 33 phenotypes for virulence to 6 sorghum entries were identified. Every year 22 to 25 virulent aphid phenotypes were being identified. Twelve phenotypes (11, 13, 21, 23, 30, 31, 32, 33, 51, 71, 72, and 73) were found every year whereas seven clones of phenotypes 20, 42, 50, 52, 56, 57, 60 were unique to this population during 4 years. It is interesting that the density of the greenbug population on sorghum did not influence intrapopulation polymorphism of the insect. Thus at outbreak of the aphid (2002 yr.), we revealed 23 virulent phenotypes and in period of the strongest depression (2003), 22 virulent phenotypes of the insect.

In studies in 1994-2001 [9, 10], phenotype 71 dominated; it possessed virulence to sorghum samples Sarvasi and Shallu widely used in breeding programs in Russia and USA. In two first samples of the greenbug in 2002 phenotype 71 dominated too, but in August its

Table 1 Phenotypic diversity for virulence in Krasnodar greenbug subpopulations.

Date of aphid sampling		Number of investigate clones	Number of virulent phenotypes	Predominate phenotype for virulence	Predominate phenotype frequency	Average number of phenotypes ($\mu \pm S_m$)	Share of rare Phenotypes ($H \pm S_h$)
2002	Jun.	58	14	71	0.24	11.34 ± 0.72	0.19 ± 0.05
	Jul.	57	18	71	0.20	11.60 ± 1.14	0.36 ± 0.01
	Aug.	52	15	10	0.21	12.48 ± 0.78	0.17 ± 0.01
	Sept.	5	4	72, 73 *	0.40	3.90 ± 0.28	0.03 ± 0.07
2003	Jun.	56	17	13	0.20	14.66 ± 0.78	0.14 ± 0.05
	Jul.	11	6	13	0.27	2.11 ± 0.86	0.65 ± 0.14
	Aug.	45	13	11	0.20	11.35 ± 0.65	0.13 ± 0.05
	Oct.	18	8	33	0.50	6.53 ± 0.73	0.18 ± 0.09
2004	Jun.	37	16	73	0.27	13.70 ± 0.56	0.14 ± 0,06
	Jul.	37	16	23	0.14	14.56 ± 0.60	0.09 ± 0.05
	Aug.	16	12	33, 73 *	0.19	9.70 ± 0.68	0.19 ± 0,10
2005	Jun.	28	12	3	0.25	11.31 ± 0.59	0.13 ± 0.06
	Jul.	37	11	33	0.38	9.12 ± 0.44	0.17 ± 0.06
	Aug.	43	16	33	0.40	11.64 ± 0.41	0.27 ± 0.07
	Sept.	17	5	3	0.41	4.55 ± 0.49	0.09 ± 0.07

* means both phenotypes have equal frequencies within a subpopulation.

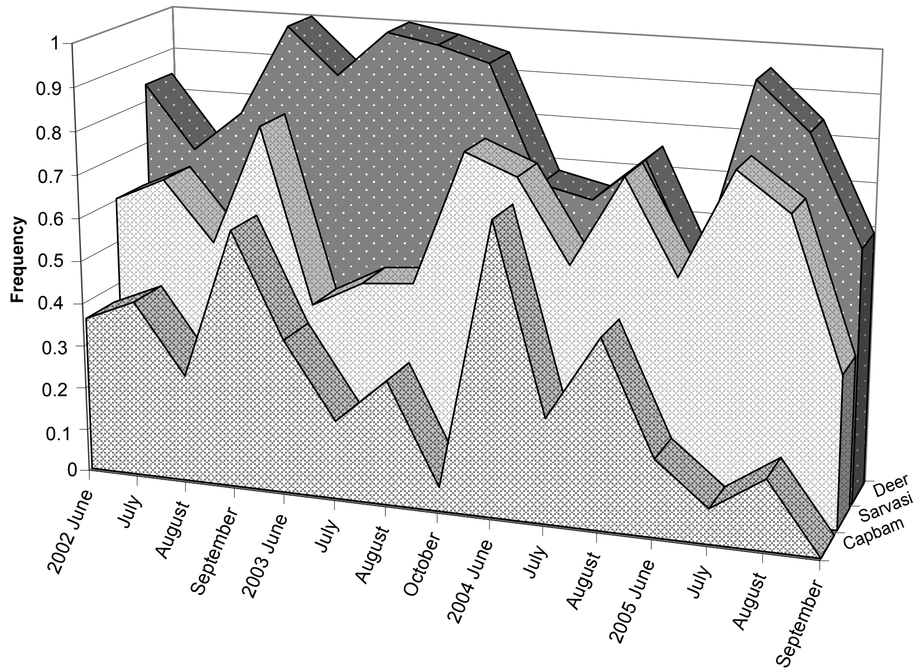

Fig. 1 Frequencies of greenbug clones virulent to the first set of differentials during four years.

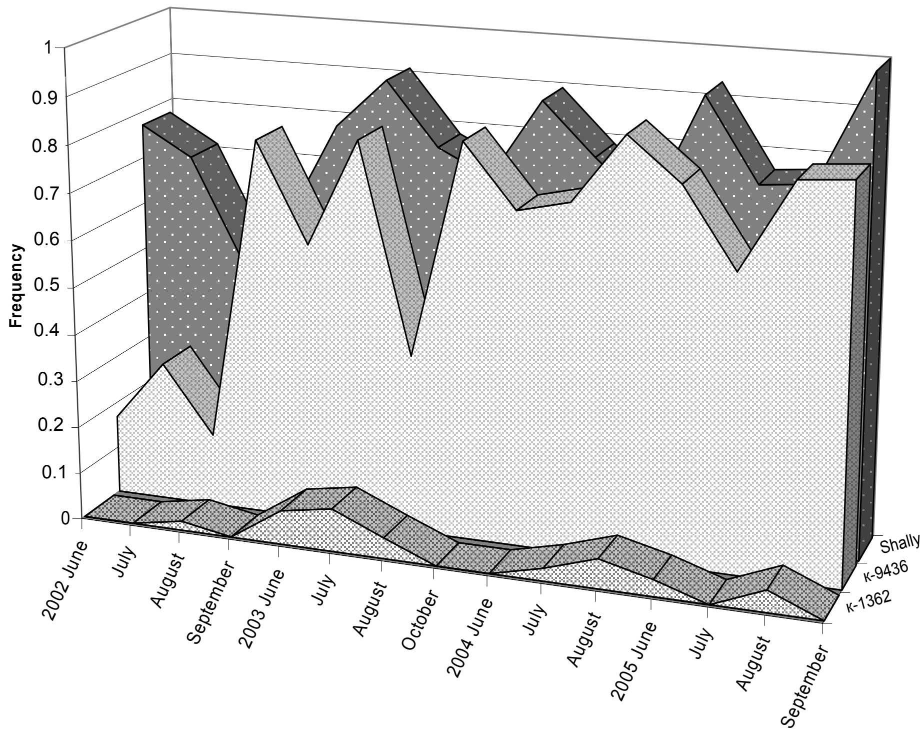

Fig. 2 Frequencies of greenbug clones virulent to the second set of differentials during four years.

frequency was very low (0.058) (Fig. 3). It should be noted that in previous experiments we compared virulence of clones sampled just in Augusts. In 2003-2005 years phenotype 71 was not dominate (Table 1, Fig. 3), although it was noted each year. Phenotype for virulence 33 was absent in sample of June, 2002, then its frequency increased and in sample August, 2005 it evidently dominated (Table 1, Fig. 3).

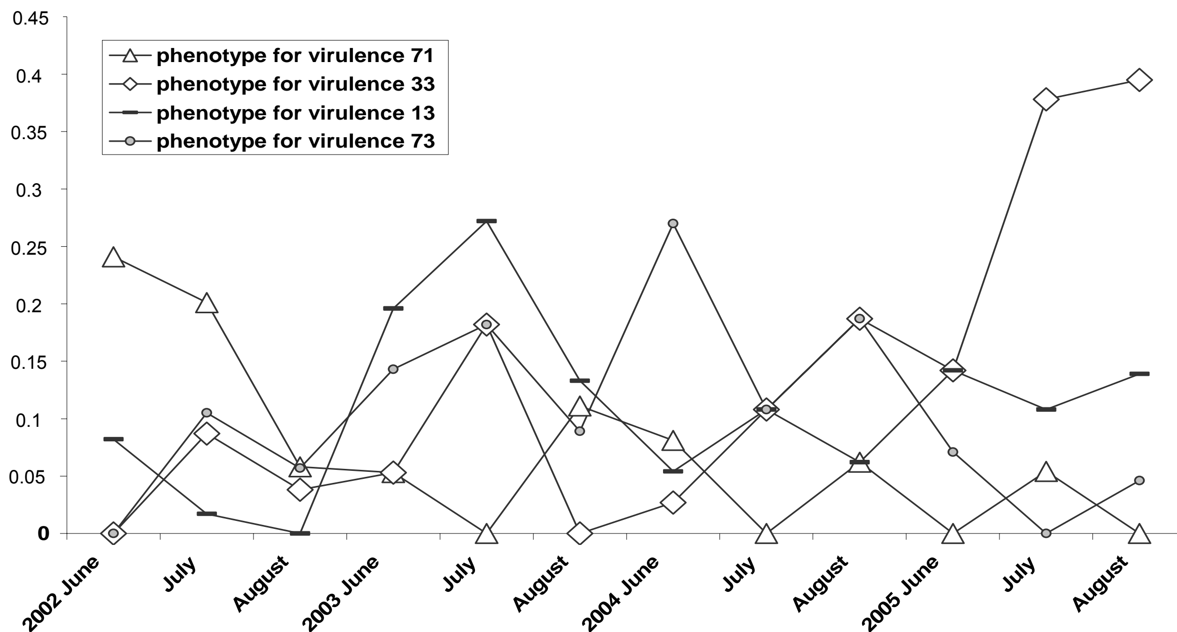

Fig. 3 Frequencies of the most common greenbug virulent phenotypes in summer subpopulations during four years.

There were slight differences between summer subpopulations for average number of phenotypes, i.e. degree of variability in them was approximately equal. Because share of rare genotypes differences among 3 samples were frequently significant especially in 2002 and 2003, June and August samples were more equaled for phenotype frequencies than July one (Table 1).

3.2 Similarity of Subpopulations

The criteria for similarities indicated that significant differences between some samples were found and varied from 0.268 to 0.739 (Table 2). Comparisons between most of subpopulations showed low similarities but likely due to that the small number of clones in the samples criterions of identity were not significant.

Comparisons of all summer subpopulations of 2002 -2005 years revealed significant differences in common virulent phenotype frequencies in 44 cases out of 66. Differences between fall samples were not found. We also compared June and fall subpopulations and found significant difference between subpopulations collected in fall of 2003 and in June of 2004 (r = 0.48; I = 35.11; $P < 0.01$).

Table 2 Similarity of Krasnodar greenbug subpopulations.

Greenbug subpopulations compared		Criterion of similarities (r)	Criterion of identity (I)
2002	Jun.-Jul.	0.661	47.92**
	Jun.-Aug.	0.702	42.57**
	Jun.-Sept.	0.268	15.83
	Jul.-Aug.	0.669	61.32**
	Jul.-Sept.	0.380	12.27
	Aug.-Sept.	0.380	12.27
2003	Jun.-Jul.	0.739	11.31
	Jun.-Aug.	0.712	32.14
	Jun.-Oct.	0.554	31.36
	Jul.-Aug.	0.508	18.14
	Jul.-Oct.	0.626	12.90
	Aug.-Oct.	0.392	32.84*
2004	Jun.-Jul.	0.576	34.73*
	Jun.-Aug.	0.657	19.14
	Jul.-Aug.	0.499	21.16
2005	Jun.-Jul.	0.524	33.59*
	Jun.-Aug.	0.621	28.16
	Jun.-Sept.	0.728	12.72
	Jul.-Aug.	0.774	20.39
	Jul.-Sept.	0.602	19.27
	Aug.-Sept.	0.536	23.57

*Difference between subpopulations is significant at $P < 0.05$; **$P < 0.01$.

4. Discussion

Even at very limited samples of greenbug clones from the Krasnodar population collected in one field (not more than 1 hectare in area) were very high overall and seasonal polymorphism of the insect for virulence was revealed. Evident change in population structure of the greenbug within one season leaded to significant skepticism about conclusions on populations' similarities and differences made on the basis of single collections for the greenbug.

The reasons for drastic changes in population structure in short time were not clear but appeared characteristic over each season for the Krasnodar population of the greenbug. One of the possible explanations for significant change of phenotypic composition of the population during the outbreak of the insect in 2002 was due to recruitment of new phenotypes by migration. Earlier we observed the same change in 2001 year [9].

The second theoretical explanation for changes in the population structure could be meiotic recombination, but the aphid reproduces parthenogenetically during summer months.

In the absence or weak migration, the main factor of the insect local population variation may be the influence of host plant genotype i.e. selection due to better adaptation just to this genotype. For this, large areas of genetically uniform varieties of the crop are necessary. In 1994-2000, 82-88% clones were virulent to Sarvasi, in 2001 their share decreased to 51% [9] that can be explained by decreasing of areas under varieties and hybrids with genes for resistance *Sgr*1+*Sgr*2 and ousting of clones virulent to Sarvasi with avirulent ones that are more fit on susceptible varieties. But in this study significant influence of selection for virulence on the host is unlikely, because we collected the aphid on sorghum field containing about 2000 entries of different origin, which are mainly susceptible to the aphid produced annually. We collected aphids from different susceptible sorghum entries. Host diversity can not explain evident changes in population genetic structure of the aphid during sorghum vegetation season, because plant diversity was constant but phenotypic diversity in the aphid population was not constant.

The data showed the key role of abiotic environmental factors in seasonal dynamics of genetic structure of the greenbug population. As additional argument for effect of abiotic factors to variability of genetic structure of *S. graminum* population collected on sorghum, we found differences between samplings for virulence to barley genotypes, i.e. the crop that is not a host for the aphid at the time of collecting (data not published).

Unfortunately data on this effect are practically absent in literature. We know the single work on differences of photoperiodic responses in *S. graminum* biotypes C and E, differs in virulence to cereal samples: minimal duration of scotophase to induce males of biotype E is slightly larger, i.e. viviparae are produced longer in fall [17].

In this work, at the end of September-beginning of October, aphid phenotypes virulent to variety Sarvasi predominated (75-80%), although for instance in 2003 frequencies of virulent clones were 0.38, 0.45 and 0.47 in 1st, 2nd and 3rd summer collections respectively. In this case, different photoperiodical responses could be of importance: to induce amphigonic generation aphid phenotypes virulent to Sarvasi requires less day length. Such environmental factors as temperature and air moisture can have even greater importance not only in plant vegetation period but even during the insect overwintering.

Certainly further investigations of ecological and genetic regulations of greenbug and other harmful organisms' population formation are necessary. Present-day notions on genetic structure of diseases causal agents' and pests' populations, degrees of different populations' isolation, probable frequencies of migrants between them, frequencies of mutations for certain genes for resistance are created in most cases after single analysis of insects and pathogens samples from certain region. However, results of our work show

that characteristics of populations for virulence frequencies can not be regarded as stable during the season.

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Study on Larval Diapause Development of *Kermes Quercus* (Linnaeus) (Hemiptera: Kermesidae) in Warsaw, Poland

Elżbieta Podsiadło

Department of Zoology, Warsaw University of Life Sciences SGGW, Ciszewskiego 8, Warsaw 02-786, Poland

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Abstract: This research presents new information on the biology of *Kermes quercus* (L.). Observations were carried out in 2009 in the city of Warsaw and surroundings (Poland). Material was collected on *Quercus robur* L. between the end of January until the beginning of November. The sampling each month was divided into three ten-day periods: I - from the 1st till 10th day of the month, II - from 11th till 20th day of the month, and III - from 21st till 30th day of the month. The material was sampled at least once in each period. All live specimens of *K. quercus* were picked off and the developmental stages were identified. The observations revealed that, almost exclusively 1st-instar nymphs occurred during the winter and they remained in this stage throughout the spring and summer until the last third of August. The process of moulting from 1st instar to 2nd instars started in the last third of August and lasted about 2 weeks. In autumn the 2nd-instars nymphs entered the winter diapause. The results suggest that, under the climatic conditions of Poland, *Kermes quercus* L. is not a univoltine species. Each generation probably develops through two years - in the first year overwintering as the 1st-instar nymphs and in the second year as the 2nd-instar nymphs.

Key words: Hemiptera, Kermesidae, *Kermes quercus* (Linnaeus), winter-diapause, 1st-instar nymphs, 2nd-instar nymphs.

1. Introduction

Kermes quercus (Linnaeus) is a Palaearctic species, very common in many European countries, where it can be a serious local pest of several species of oaks. It lives mainly in the bark crevices of trunk and on the thick branches but, when its density is high, it can also occur on thinner branches. In the region of St Petersburg, Nasonov observed such a high density that they almost entirely filled bark crevices and branches, causing the death of the trees [1]. Kozár also reported a mass infestation of oaks in Hungary, reaching 250 individuals per dm^2 [2]. Recently, the present author has found a high density in the Bemowo wood-park in Warsaw.

Nasonov was the first author to study the biology of *K. quercus*, in the region of St Petersburg in 1907 and 1908, but his description of the life cycle of the species was not clear [1]. Nasonov found that *K. quercus* overwintered as the 1st- and 2nd-instar but, in Nasonov's opinion, these 2nd-instars nymphs were from the previous year. He reported that, in the spring of the following year, the 2nd-instars nymphs developed into adults. However, he did not report what had happened to the first-instar nymphs.

Saakjan-Baranova and Muzafarov outlined a different life cycle from the region of St Petersburg [3]. According to these authors, *K. quercus* had only one generation per year, over wintering as the 2nd instars. In the spring, the 2nd instars moulted into adults and, after mating, the adult females started to lay eggs. 1st instar nymphs hatched from the eggs, moulting into the 2nd instars in August which then overwintered. A similar life cycle of *Kermes quercus* was reported by

Corresponding author: Elżbieta Podsiadło, Prof., research field: entomology. E-mail: elzbieta_podsiadlo@sggw.pl.

Schmutterer from Germany [4].

Such a scheme of life cycle of *K. quercus* has been generally accepted and is that given in Scale Net.

However, observations carried out in Poland have not confirmed any of the above life cycles of *K. quercus* but are closest to that given by Nasonov [1].

2. Material and Methods

The observations were carried out between the end of January and the beginning of November in 2009. The material was collected on *Quercus robur* L., almost exclusively in the Bemowo wood-park, situated in the western part of Warsaw. Only the first two samples, collected in March, were from the Kampinoski National Park near Warsaw.

The mean monthly temperatures for Warsaw in 2009 are given in Table 1.

Pieces of bark or smaller branches were cut off from the trees and all live specimens of *K. quercus* were picked off and preserved in 70% ethanol in the laboratory.

The sampling each month was divided into three ten-day periods: I - from the 1st till 10th day of the month; II - from 11th till 20th day of the month; and III - from 21st till 30th day of the month.

The materials were sampled at least once in each period.

Generally, the larval stages could be identified under a stereomicroscope after preliminary soaking the individuals in a 10% KOH solution for 24 hours. When doubts arose, the specimens were mounted on slides and examined at a higher magnification. The number of examined specimens of *K. quercus* is given in Tables 2 and 3.

3. Results and Discussion

As can be seen in Table 2, almost exclusively 1st-instar nymphs were recorded during the winter and they remained in this stage throughout the spring and summer until the last third of August (Table 3). During this winter and spring period, second-instar nymphs of both sexes were only rarely found, although the 2nd instar females that did over winter moulted into the third instar in April (Table 2). None were found in June and July (Tables 2 and 3). It is not yet known what had happened to them afterwards. Occurrence of these 2nd-instar nymphs has not been clear. Perhaps the 1st-instar nymphs moulted sporadically before the winter diapause. However, they only constituted about 1% of the individuals collected and most nymphs overwintered in the first nymphal stage.

The moult of the 1st-instar nymphs started in the last third of August and proceeded rapidly (Table 4). On the 17th August (2nd third), there were only 1st-instar nymphs but by the 27th (last third), 1st-instar nymphs constituted only 25% of the individuals collected, and by the 7th September, only 4% of the 1st instar remained.

Table 1 Mean monthly temperatures (℃) in Warsaw in the year 2009 [5].

Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
-2.7	-0.6	2.7	11.3	13.6	16.2	19.9	18.6	15.5	6.9	5.6	-1.0

Table 2 The number of specimens of *Kermes quercus* (L.) collected on *Quercus robur* L. in Warsaw and surroundings between January and June 2009.

	Month: 10 day periods															
	Jan.	Feb.			Mar.			Apr.			May			June		
	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
L_1	45	149	110	75	293	305	120	192	140	192	120	100	212	100	120	246
L_2♀	1	1	0	0	0	5	0	2	0	0	0	0	0	0	0	0
L_2♂	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
L_3♀	0	0	0	0	0	0	0	0	5	1	0	0	0	0	0	0

Table 3 The number of specimens of *Kermes quercus* (L) collected on *Quercus robur* L. in Warsaw between July and November 2009.

	Month: 10 day periods												
	July			Aug.			Sept.			Oct.			Nov.
	I	II	III	I	II	III	I	II	III	I	II	III	I
L_1	130	318	225	140	140	36	6	18	10	13	5	4	4
L_2♀	0	0	0	0	0	65	121	315	183	280	248	257	158
L_2♂	0	0	0	0	0	41	17	11	40	84	11	21	57
L_3♀	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4 Development of *Kermes quercus* (L.) on *Quercus robur* L. between August and November 2009 in Warsaw. Number of specimens is given as %.

	Month: 10 day periods									
	Aug.			Sept.			Oct.			Nov.
	I	II	III	I	II	III	I	II	III	I
L_1	100	100	25	4	5	4	3	2	1	2
L_2♀	0	0	46	84	92	79	74	94	91	72
L_2♂	0	0	29	12	3	17	22	4	7	26

Clearly, the process of moulting from 1st instar to 2nd instars lasted about 2 weeks. Only single 1st-stage nymphs were seen to moult in the middle and end of September and in the beginning of October. No moulting was observed from the middle of October and in November. Clearly, the remaining 1-2% of 1st -instar nymphs stopped growing and would probably die during the winter. So, *K. quercus* entered the winter diapause in Autumn of 2009 as the 2nd larval stage.

4. Conclusions

(1) Under the climatic conditions of Poland, *Kermes quercus* (L.) is not a univoltine species.

(2) Each generation probably develops over two years. In the first year individuals overwinter as the 1st-instar nymphs and in the second year as the 2nd -instar nymphs.

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Molecular Evolution of the Cytochrome *b* Gene in Geographically Isolated Populations of *Oryzias Latipes*

Nobuhiko Asada[1], Akira Hirai[2], Taiki Hamada[1], Eri Sato[1], Akio Moriyama[3] and Tetsuro Takeuchi[4]

1. Department of Zoology, Faculty of Science, Okayama University of Science, Kita, Okayama 700-0005, Japan

2. Misaki-cho, Okayama 716-1122, Japan

3. Hamada 3rd Junior High School, Hamada 695-0101, Japan

4. Faculty of Humanities, Shujitsu University, Higashi, Okayama 703-8255, Japan

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Abstract: The mitochondrial cytochrome *b* gene was isolated from the caudal fin of *Oryzias latipes* and PCR was undertaken to determine phylogeny. The nucleotide sequence of the complete cytochrome *b* gene including the 5' and 3' ends was 1,143 base pairs (bp) and 1,137 bp encoding 380 and 378 amino acids in the K-11 strain and HS strain, respectively. In addition, higher substitutions both in base and amino acid residues occurred more frequently between the former control (Hd-rR strain) and HS strains than the K-11 strain. Approximately similar values in polymorphism, as assessed by restriction endonuclease digestion, were detected when utilizing 20 different enzymes. Therefore, the evolutionary processes were unlikely to involve common ancestors, especially between the K-11 strain and HS strains, in *O. latipes* populations in Japan. The sequence had been deposited in GenBank Data Base under accession number AB480878 (K-11 strain) and AB480879 (HS strain).

Key words: Cytochrome *b*, RFLP, diversity, evolution, *Oryzias latipes*.

1. Introduction

In recent years, a number of studies have investigated genetic variation in mitochondria DNA (mtDNA) within and between species and natural strains. A crucial finding arising from these studies is that mtDNA exhibits a relatively high rate of nucleotide substitution compared to nuclear DNA, and thus it is well suited for the study of genetic divergence in closely related species and geographically isolated strains. Killifish, (*Oryzias latipes* Jordan and Snyder, 1906), is an endemic fish species to all of the islands of Japan, except for Hokkaido located in the northern part of Japan. In the Yedo era (1603-1867) in Japan, *O. latipes* was a familiar fish and was widely depicted in the traditional *ukiyoe* woodblock prints.

Corresponding author: Nobuhiko Asada, Ph.D., Prof., research field: population genetics. E-mail: asada@das.ous.ac.jp, asada@zool ous.ac.jp.

Analysis of mtDNA serves as a powerful molecular marker for the reconstruction of evolutionary lineage in vertebrate species including fish [1]. The cytochrome *b* gene is located within the mitochondria gene, indicating its ancient origins, and is one of the most frequently utilized segments of mtDNA as it is relatively easy to align and it has been extensively characterized in numerous vertebrates. The *O. latipes* strain (K-11) (belonging to sub clade B-VIII [2]) is a member of the Western Seto-Inland population and belongs to the Southern population [2]. K-11 appears to have been geographically isolated from the endemic population and for a long time was only distributed within a small pond in the middle part of the Okayama Prefecture in Japan. A second strain, the HS strain (belonging to sub clade B-X [2]) is a member of the San-in population and also belongs to the Southern population. The HS strain resides in a small canal in the western part of the Shimane Prefecture in Japan.

These two natural populations of *O. latipes*, the Western Seto-Inland and San-in populations, demonstrate a vastly different distribution and are exposed to quite contrasting climate conditions and local ecosystems. The Western Seto-Inland population is exposed to warm and dry climate conditions, while the San-in population is subjected to a cooler and higher humidity climate. Biodiversity of *O. latipes* is primarily drived by natural selection processes including diversification, balancing, critical mutation and stochastisity. In this study, we analyzed cytochrome *b* gene expression in the Japanese endemic population of *O. latipes*.

2. Materials and Methods

2.1 Extraction of mtDNA

For the extraction of mtDNA from the K-11 and HS strains, the posterior region of the caudal fin was dissected using a surgical knife and mtDNA from a single discarded fin was extracted using the method previously reported [3] with some minor modifications. Briefly, a single fin fraction was ground in 100 µL homogenizing buffer solution containing 250 mM sucrose, 10 mM EDTA, 2.5 mM $CaCl_2$, 30 mM Tris-HCl (pH 7.5) and 1 µL of 10 mg/mL proteinase K solution at 25 ℃, and incubated at 55 ℃ for 60 min. After incubation, an equal volume of buffer-saturated phenol chloroform-isoamylalcohol (24:1) was added and centrifuged at 16,000 rpm for 5 min at 4 ℃. The precipitate containing the mtDNA was then dissolved in sample buffer containing 0.5 mM EDTA and 5 mM Tris-HCl (pH 7.5 at 25 ℃), and stored at –25 ℃ until required.

2.2 PCR Conditions

mtDNA encoding cytochrome *b* were amplified by PCR using specifically designed oligonucleotide primers. Sense and anti-sense oligonucleotide primers were constructed based on the methods previously described [2]. PCR was carried out in 50 µL amplification reaction mixture containing 25 pmol of each primer and 1.23 units Taq DNA polymerase. PCR conditions were as follows: 40 cycles of denaturation at 94 ℃ for 30 sec., annealing at 55 ℃ for 30 sec., and extension at 72 ℃ for 30 sec.. The amplification reaction was undertaken in a Parkin Elmer Gene Amp Systems 2400 (Applied Biosystems, Foster, CA, USA), and the amplified PCR product was subjected to electrophoresis on a 1.5 % agarose gel (LO3, Takara Co., Kyoto, Japan) and stained with ethidium bromide. The PCR product was then purified using a spin column (Quantum Prep, Bio-Rad Laboratories, Hercules, CA, USA) and the DNA was sequenced.

2.3 Sequencing of the Cytochrome b Nucleotide

The nucleotide sequence was obtained using the dye-terminating method [4] and a Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit (GE Healthcare, Buckinghamshire, UK) in a SEQ4x4 Personal Sequencing System (Amersham Pharmacia Biotech UK Limited, Uppsala, Sweden). Sequence data was analyzed using the computer software Clustal W and Genetyx (version 9).

2.4 Restriction Fragment Length Polymorphism (RFLP) Analysis

The sites of nucleotide sequences of cytochrome *b* were estimated and can with 20 restriction endonucleaes, *Bam* HI, *Eco* RV, *Hae* III, *Hpa* II, *Mbo* I, *Msp* I, *Pst* I, *Rsa* I, *Sau* 3AI and *Taq* I were used for the RSP analysis. Each distinct restriction fragment pattern produced by any of the 10 enzymes was assigned an uppercase letter code in alphabetical order of the detection.

3. Results and Discussion

3.1 Sequence of Nucleotide and Amino Acid Residues

We identified PCR-based multiple fragment patterns including the entire cytochrome *b* gene. The composite cytochrome *b* sequence was 1,143 nucleotides in length for the K-11 strain and was 1,137 nucleotides in length for the HS strain, and included the first nucleotide of

the ATG initiation codon and extended to the TAA terminus codon. The total number of deduced amino acid residues was 380 and 378 in K-11 and HS strains, respectively. These numbers were approximately equal to the former control Hd-rR strain that was 1,147 nucleotide and 380 amino acid residues in length.

3.2 Divergence of Nucleotide and Amino Acid Residues

The total number of nucleotide and amino acid residue substitutions in the cytochrome *b* gene was estimated using nonparametric methods, in order to isolate closed circular mtDNA digestion. The mathematical theory can be used to study the rate of nucleotide substitution, which also serves as a basic quantity in the study of molecular evolution. The rate of nucleotide substitution was based on the number of nucleotide differences in each pair, and when calculated was shown to be twice that in the HS strain compared to the Hd-rR and K-11 strains (Table 1). The total number of amino acid residue substitutions was also calculated using the same methods, and revealed a similar divergence between the strains (Table 1).

In addition, the rate of both synonymous and non-synonymous substitutions demonstrated large variations among the three strains. Non-synonymous substitution values were approximately 0.789, suggesting that higher isolation is currently occurring among the Southern populations. The values for the K-11 and HS strains were approximately 0.72 (Table 2), a value similar to that of erythropoietin [5]. For the cytochrome *b* gene, the ratio of non-synonymous (Ka)

Table 1 Rate of nucleotide and amino acid substitutions.

Strain	Nucleotide	Amino acid
Hd-rR vs. K-11	0.02	0.07
Hd-rR vs. HS	0.29	0.37
K-11 vs. HS	0.23	0.34

Table 2 Rate of synonymous and non-synonymous substitutions.

Strain	Synonymous	Nonsynonymous
Hd-rR vs. K-11	0.811	0.188
Hd-rR vs. HS	0.419	0.580
K-11 vs. HS	0.211	0.789

to synonymous (Ks) divergence should be equal to one (Ka value/Ks value), but selective constraints may result in a lower ratio.

In order to assess the levels of selective constraints, we calculated Ka values/Ks values and found that comparisons between Hd-rR strain vs. K-11 strain, Hd-rR strain vs. HS strain and K-11 strain vs. HS strain, Ka values /Ks values were 0.232, 1.384, and 3.739 were obtained respectively. These values indicate selective removal of deleterious non-synonymous mutations in the cytochrome *b* gene of the two *O. latipes* natural populations. In addition, if nucleotide substitution occurs at random within the three positions of the codon, the proportion of nucleotide substitution may be estimated from the genetic code.

3.3 Pattern of RFLP

Restriction sites digested with 20 different restriction endonucleases demonstrated polymorphisms in both the K-11 and HS strains in *O. latipes* mtDNA, and revealed that they were approximately equal to the Hd-rR former control strain (Fig. 1). The deduced cut sites of several restriction endonuclease demonstrated that there were almost no typical phenotypic differences between the K-11 and HS strains. With respect to the presence or absence of each restriction site, three different mtDNA haplotypes were recognized in *O. latipes*, and each haplotype between the strains differed the gain or loss of sites. Polymorphism in the cleavage fragment patterns revealed by the restriction pattern of several enzymes was tentatively designated as type (Fig. 1).

The π value for the three strains was estimated to be 0.072, and appeared to indicate that π_{dH} was twice as diverse as π_{dK} in terms of distribution. In regard to evolutionary rates, the divergence time was estimated for sub clades B-I to B-XI and ranged from 0.5 to 2.3 million years when based on paleo-geographic data [2]. According to the calculated π values, the total number of nucleotide substitutions per year for the three strains was estimated to be approximately 2.3 million year

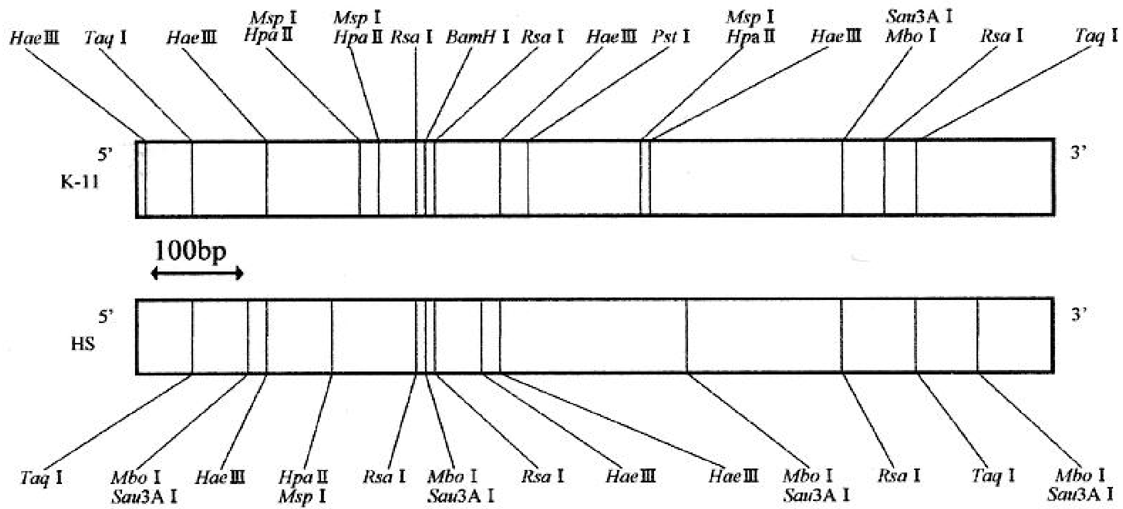

Fig. 1 RFLP patterns of cytochrome b gene in *Oryzias latipes*. Above and below shows K-11 strain and HS strain, respectively.

ago. As a result, it is suggested that the HS strain is currently undergoing molecular and phenotypic incipient evolution.

3.4 Evolution of Oryzias Latipes

The origin of the Western Seto-Inland and San-in populations has been suggested to be the northern Kyusyu area located on the western-most island of Japan [2]. The evolution of the K-11 and HS strains, whose habitats are geographically isolated on the opposite slopes of the Chugoku Mountains in Japan, significantly differ from each other in terms of geographic selection. These differences are in line with the southern coasts of lowland Seto Inland Sea versus the steep in face with the Korean Peninsula and Japan Sea through random genetic drift. Previous reports [6] speculated from the phylogeny of *O. latipes* that the “Jomon-peaple”, Shiba dog (the San-in small Japanese endemic Shiba dog) [7], and Tsushima wild cat all migrated from Central Asia to the San-in area of Japan via the Korean Peninsula. Thus, random genetic drift may occur via various routes from the Korean Peninsula, the northern Kyusyu area and the San-in natural populations. Given the prevalence of *O. latipes* in the rice fields of Japan, and the similarity in the taxonomic names of *O. latipes* and Japanese rice *Olyza sativa*, it has long been suggested that *O. latipes* immigrated to Japan along with Japanese rice. However, *O. latipes* appears to have been distributed from the continental clade to Japan 5.4-6.0 million years ago in the Paleolithic era [2] and has only become associated with Japanese rice over the last 300,000 years or so. Thus, we propose that *O. latipes* was not introduced to Japan along with rice and was instead introduced at a much earlier time point.

4. Conclusions

The genetic diversity and genome-phenome organization in *Oryzias latipes* have been explored by using molecular techniques. The main genetic difference cause seems to be geographic isolation between the small freshwater fish, *Oryzias latipes*. The co-origin of the K-11 strain and HS strain is estimated at the northern Kyushu district, one of the southern islands of Japan, then genetic diversify to the north, the HS strain, or the south, the K-11 strain, by the effect of emigration and stochasticity.

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PROD and BROD Modulation in Nile Tilapia after Exposure to 17 β-estradiol

Franciele Bitencourt[1], Rosa Toyoko Shiraishi Frighetto[2], Júlio Ferraz de Queiroz[2], Marcos Eliseu Losekann[2], Alfredo Jose Barreto Luiz[2] and Eduardo Alves de Almeida[1]

1. Departament of Chemistry and Environmental Sciences (IBILCE), São Paulo State University, São José do Rio Preto, São Paulo 15054-000, Brazil

2. The Brazilian Agricultural Research Corporation (EMBRAPA), São Paulo 13820-000, Brazil

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Abstract: The activities of 7-ethoxyresorufin-*O*-deetylase (EROD), 7-benzyloxyresorufin-*O*-debenzylase (BROD), 7-pentoxyresorufin-*O*-depentilase (PROD), and glutathione S-transferase (GST) were measured in Nile tilapias exposed for 7 days of 5 and 15 μg/L 17 β-estradiol. EROD and GST activities were unchanged. PROD activity increased in animals exposed to the higher dose of the hormone, while BROD was increased after 7 exposure days to both doses of the compound. These results indicate the usefulness of these enzymes as biomarkers for 17 β-estradiol exposure.

Key words: EROD, PROD, BROD, 17 β-estradiol, biomarker, Nile tilapia.

1. Introduction

Endocrine disruptors (EDs) can be defined as exogenous agents that interfere with the synthesis, secretion, transport, reception, action or the elimination of endogenous hormones in organisms [1]. Among the most important endocrinal disrupters found in aquatic environments are natural and synthetic estrogens, because they are extremely active at very low concentrations and are related to the etiology of numerous cancers [2, 3]. Natural estrogens like 17 β-estradiol, estriol, estrone and synthetic estrogens, developed for medical purposes [4], are among the most important concern, due to their high potency and the continuous use, with an increasing input in the environment. A significant amount of estrogens are excreted by humans in domestic wastes, being the human and animal excretion the main source of EDs in the aquatic environment [2, 3, 5].

Corresponding author: Eduardo Alves de Almeida, Ph.D., assistant professor, research field: ecotoxicology. E-mail: ealmeida@ibilce.unesp.br.

The simple detection of EDs in water is not a simple approach, since exhaustive extraction procedures and expensive methods are sometimes needed [6]. Alternatively, the measurement of biochemical biomarkers can sometimes elicit the exposure of aquatic animals to EDs without the need of sophisticated chemical analyses.

One of the most common approaches for the evaluation of EDs effects in aquatic animals is the analysis of vitellogenin, precursors of the egg-yolk proteins [7]. Vitellogenin synthesis occurs only in mature females in response to endogenous estrogens [8], but in males, the vitellogenin genes becomes active in the presence of xenoestrogens [9], therefore being excellent indicator of exposure to EDs [10]. Among vitellogenin measurement, the search for alternative biomarkers for ED exposure in the aquatic environment could improve the options for aquatic biomonitoring studies.

The aim of this work was to investigate how the activities of some cytochrome P450 isoforms from

liver of male Nile tilapia (*Oreochromis niloticus*) respond to low concentrations (5 and 15 μg/L) of 17 β-estradiol (E2) in water after seven days of exposure. Despite this hormone is usually found at ng/L level in most studies in the literature, it was found at 3 to 5 μg/L in the water resource of Campinas city (São Paulo, Brazil), thus justifying the concentrations tested in the present work [3].

2. Methodology

2.1 Exposure to E2

Twenty-seven male fish were separated into nine aquarium (3 fish per aquarium) containing 115 L of filtered, dechlorinated and aerated water. The fish remained at this condition for one week, then the hormone E2 was added to six of the nine aquariums at 5 μg/L (3 aquariums) and 15 μg/L (3 aquariums), composing three replicates for each group (N = 9): control, exposed to 5 μg/L and exposed to 15 μg/L. After seven days of exposure to the hormone, all fish were killed and had their gills and liver collected for enzymatic analyses. The average length and weight of the animals used in the experiment are presented in Table 1. Tilapia is generally considered adult by aquacultors after surpassing 30 g [11]. As the fish used in our experiments were among 190 g, they were considered all mature fish.

2.2 Biochemical Analyses

In liver, the activities of 7-ethoxyresorufin-*O*-deetilase (EROD), 7-benzyloxyresorufin-*O*-debenzylase (BROD), 7-penthoxyresorufin-*O*-depentilase (PROD) and glutathione *S*-transferase (GST) were evaluated, and in the gill only GST was measured. The tissues were weighed and homogenized with 5 volumes of buffer (Tris 50 mM, 0.15 M KCl, pH 7.4), and centrifuged at 10,000 g for 20 min at 4 ℃. The supernatant fraction was centrifuged at 55,000 g for 60 min at 4 ℃, in order to obtain the cytosolic and the microsomal fraction. The activity of GST was measured in the cytosolic fraction, while the activities of EROD, PROD and BROD were measured in the microsomal fraction.

Table 1 Values of length and weight (media ± standard deviation) of tilapias used in the exposure experiment.

	17 β-estradiol (E2) (μg/L)		
	0	5	15
Length (cm)	15.0 ± 1.1	14.6 ± 1.3	13.9 ± 1.5
Weight (g)	193.3 ± 48.8	191.7 ± 47.8	193.8 ± 43.5

The attribution of EROD, PROD and BROD to specific phase I biotransformation enzymes is controversial in the literature. The activities of EROD and PROD were generally referred to CYP1A and CYP2B, respectively, in fish and mammals [12]. In mammals BROD activity showed broader substrate specificity and was a known marker for CYPs 1A, 2B and 3A [13]. The activities of these CYP subfamilies were measured by the fluorimetric method described by Burke and Mayer [14], with some modifications. The substrates 7-ethoxyresorufin, 7-benzyloxiresorufin and 7-penthoxyresorufin were used in the assay of EROD, BROD and PROD, respectively, at a final concentration of 5 μM, and the assay was recorded (excitation 535 nm, emission 590 nm) during three minutes in the presence of 20 μM of NADPH. The activity of GST was determined by the increasing in absorbance at 340 nm, using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates, according to Keen et al. [15]. The total protein content was determined by method of Bradford [16].

2.3 Statistical Analyses

Control and treated animals were statistically compared by the Student *t* test, by means of the Microcal Origin 6.0 (Northamptom, MA, USA), and just $P < 0.05$ was accepted as significant.

3. Results

No mortality was observed along the experiment. The activities of EROD and GST were unchanged comparing control and treated groups (Fig. 1). On the other hand, the activity of BROD was increased after seven exposure days to 5 μg/L and 15 μg/L of E2.

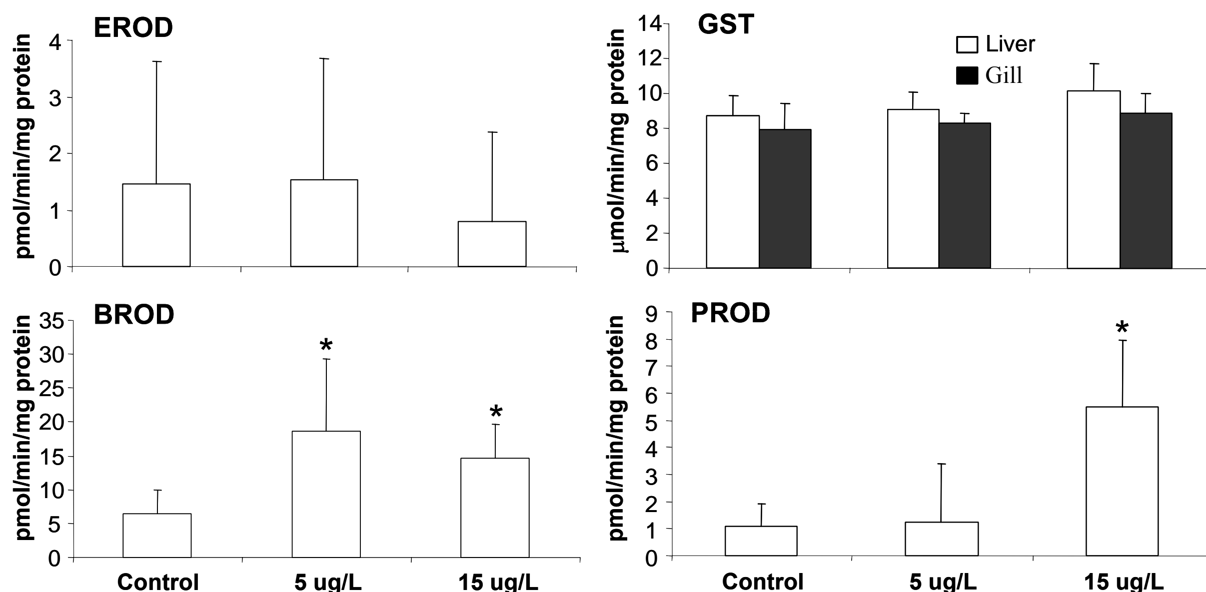

Fig. 1 Activities of BROD, PROD and EROD in the liver and of GST in the liver and gills of Nile tilapias exposed for 7 days to 5 and 15 μg/L of 17 β-estradiol. *indicates statistical differences between related to control value ($P < 0.05$).

PROD activity was increased only in those animals exposed to the higher E2 dose.

4. Discussion

The present study preliminarily determined the responsiveness of GST, EROD, BROD and PROD activities to E2 in Nile tilapia. A number of reports have indicated that the suppression of the hepatic CYP1A and associated EROD activity occur in various male or female fish after experimental treatment with E2 or other xenoestrogens [17-20], although investigations into the effects of this hormone on other CYP isoforms in fish are scarce. Although not statistically different, we observed a trend of EROD activity inhibition in fish exposed to higher E2 dose.

EROD activity is generally related to CYP1A family, which are most related to polyaromatic hydrocarbons and polychlorinated biphenyls exposure, being not related to estrogen metabolism. Indeed, according to Matozzo et al. [7], most of hormones including E2 were conjugated with glucuronides before their excretion, thus GST could not be the main enzyme activated for E2 conjugation. Indeed, Hughes and Gallager [21] demonstred that exposure of largemouth bass (*Micropterus salmoides*) to E2 (2 mg/kg) did not affect steady-state GST-A mRNA expression in liver, despite GST activity increased after 2 days of exposure to E2, using CDNB as substrate. It could be possible that GST increased as well in tilapias from our experiments after 2 days of exposure, but fish were collected only after 7 days of exposure, and this remains to be confirmed.

On the other hand, the activities of BROD and PROD were very sensitive to this hormone, being increased after seven days of exposure to 5 (BROD) and 15 μg/L (BROD and PROD) of E2. This strongly suggests the involvement of these enzymes in metabolism of the hormone. It is interesting the fact that these two enzymes increased strongly after exposure to very low levels of the contaminant, indicating their high sensibility to this hormone and their potential use as exposure biomarkers for the presence of E2 at μg/L levels in water. Regardless of CYP isoform specificity of the substrate used for the activity of P450 in exposed fish, the present study demonstrated that E2 exposure modulated the activity levels of these putatively different CYP subfamilies. Further studies should be done with less E2 concentration and longer exposure periods, since most of environmental studies had detected this hormone at

ng/L levels in the aquatic environment [22].

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The Maximum Temperatures (T_{max}) Distribution on the Body Surface of Sport Horses

Ewa Jodkowska[1], Krzysztof Dudek[2] and Maciej Przewoźny[3]

1. Department of Horse Breeding and Riding, Wrocław University of Environmental and Life Sciences, Wrocław 51-631, Poland

2. Institute of Machines Design and Operation, Technical University of Wrocław, Wrocław 50-231, Poland

3. Equi Vet Serwis, Buk 64-320, Poland

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Abstract: The objective of the study was to determine the usefulness of measuring maximum temperatures in designated regions of a healthy horse's body surface. Thermographic investigations (Thermovision®550, FLIR) were carried out on 35 horses from 6 to 16 years old participating in show jumping competitions. The rectal temperatures of the horses were in the range 37.5-38.2 ℃. The research was performed in a stable, the ambient temperature (T_{ab}) of which was 14 ℃ with a humidity (φ) of 60%. Thermograms of the left and right side of the horses were obtained. Each thermogram consisted of 36 body surface regions (before the competition) and 25 regions (immediately after the competition). The maximum temperature ranges at rest were 21.8 ℃-31.0 ℃ and symmetrical regions did not differ statistically ($P > 0.05$). The highest temperatures were on the head, neck and trunk, the lowest-on the limbs. The hind legs were warmer than the front legs in analogous areas, with the exception of the gaskin and forearm. The warmest body areas had the largest surface area, which is indicative of their crucial role in the thermoregulation of the equine organism. The research results may therefore be useful in veterinary diagnosis. The range of maximum temperatures after the competitions was 25.2 ℃-34.2 ℃. The highest increment was observed at the breast, elbow, forearm and gaskin, the lowest–at the head, pastern and hoof (fore- and hind limbs). Research regarding body surface temperature after exercise does not have diagnostic value for veterinarians because "warming-up" certain parts of body surface masks inflammation.

Key words: Horses, thermographic research, maximum temperatures, body surface.

1. Introduction

Horses are homeothermic animals whose daily and seasonal changes in body temperature do not exceed ±1.5 ℃. Rectal temperature constitutes an indirect indicator. Due to their tolerance to substantial changes in ambient temperature, horses are eurythermic, which means that the amount of heat their organism produces may vary within broad limits. Minimum amounts of heat are released during necessary, physiological, life-maintaining processes, whereas maximum amounts of heat are released during long-term strenuous physical effort. A horse's organism loses a majority of its heat through its skin by means of convection, radiation, evaporation and conduction as well as via the respiratory system as a result of evaporation [1]. The loss of heat gives rise to a change in the temperature at the body's surface. Thermovision technology allows its temperature to be monitored as a result of the transformation of infrared radiation into light emission. The colours seen on thermograms correspond to temperature ranges, depending on tissue vascularization [2].

It is apparent from the above that the temperature at the surface of the horse's body may constitute an indicator of changes in thermoregulation. It must be stressed that this is modified by the influence of micro-climatic conditions, primarily ambient temperature [3].

Corresponding author: Ewa Jodkowska, Ph.D., assistant professor, research fields: ethology and welfare, physiology, feeding of horses. E-mail: ewa.jodkowska@up.wroc.pl.

Certain diseases may also give rise to changes in body temperature [4-6]. Research in this respect has been conducted by a large number of authors (since 1964), the majority of which are cited in the professor dissertation [7].

The objective of our study was to determine the usefulness of measuring maximum temperatures in designated regions of the body surface of a healthy horse in relation to the musculature of particular body parts and the determination of the warmest and the coldest regions.

2. Materials and Methods

Thermogram research was carried out on 35 horses from 6 to 16 years old participating in show jumping competitions. The body-surface temperature of each horse was registered from left and right sides. The measurements were made half an hour before the competition and immediately after its completion. A Thermovision®550 (FLIR) thermovisual camera was used to measure temperature variations from a distance of approx. 3 metres. The thermograms obtained were analysed in relation to specific regions of the body on the basis of equine anatomy. The maximum temperature was determined for each of the regions, and the warmest places were precisely identified. Temperatures in 36 regions of the horse's body were measured by thermovisual camera prior to the competition (Fig. 1).

Immediately after the competition, it was only possible to register temperatures in 25 regions of the body (due to the presence of a rider on the horse's back and pads on the limbs) (Fig. 2).

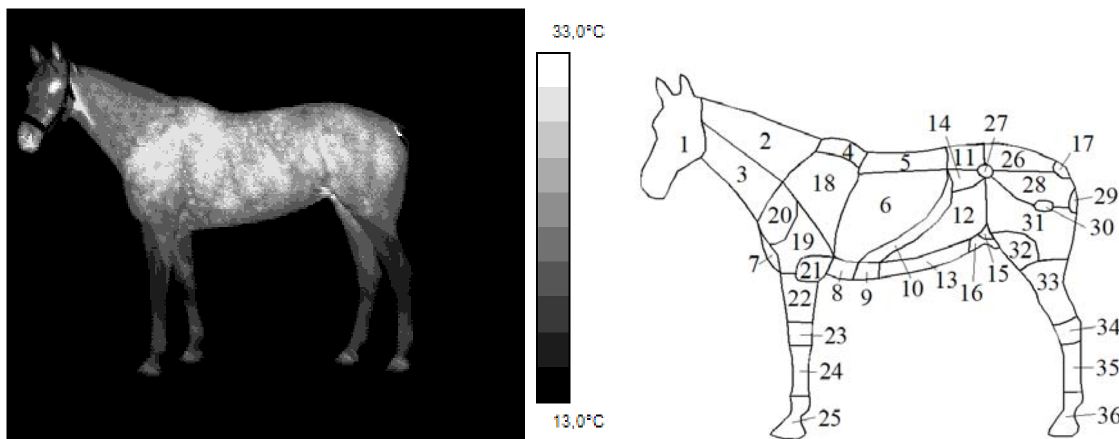

Fig. 1 Thermogram and regions of horse conformation before competition (explanation in Table 1).

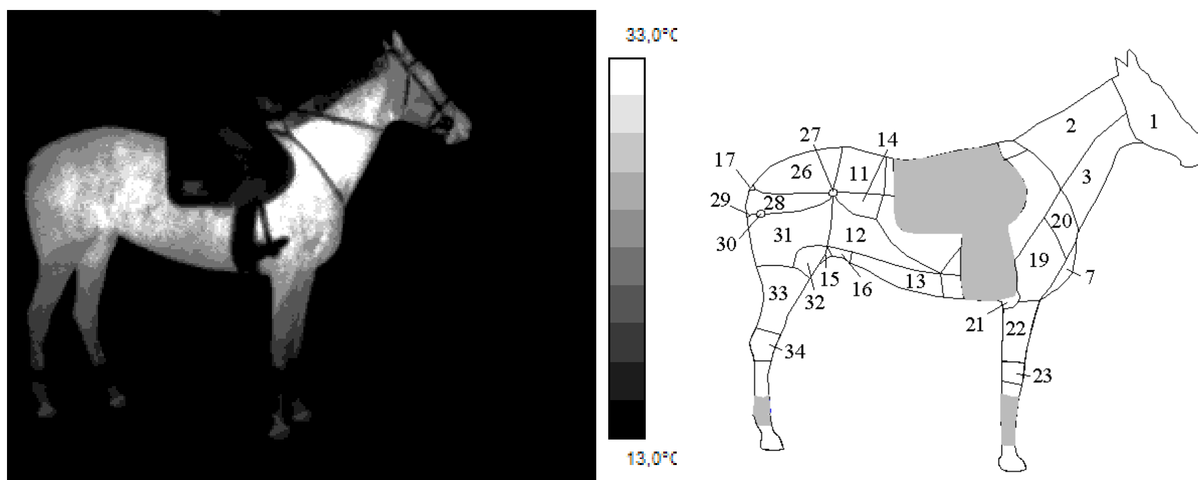

Fig. 2 The thermogram and regions of horse conformation after the competition (explanation in Table 2).

The rectal temperature of all horses fell within the boundaries of 37.5-38.2 ℃. The research was conducted in stables with an ambient temperature of T_{ab} = 14 ℃ and a relative humidity of φ = 60%.

The results of the research were analysed statistically, determining the maximum temperature and standard deviation, applying a Shapiro-Wilks normality test and a student's t-distribution test using Statistica 9.0 software of StatSoft Company (Poland) [8].

3. Results and Discussion

The maximum temperature of the horses' body surface at rest was from 21.8 ℃ to 31.0 ℃ (Table 1).

The lowest temperatures were observed in the case

Table 1 Maximum body surface temperature of horses before competition.

No.	Region of interest (ROI)	Maximum temperature ($\overline{T}_{max} \pm S_T$ (℃))		Test L v. R *P* value	Surface	
		Left side	Right side		cm^2	%
1	Head	30.8 ± 1.8	31.0 ± 1.7	0.687	1,338	8.3
2	Dorsal s. of neck	29.2 ± 2.0	29.8 ± 1.6	0.155	1,313	8.1
3	Ventral s. of neck	29.7 ± 2.4	29.6 ± 1.9	0.809	901	5.6
4	Withers	28.9 ± 2.3	29.4 ± 2.3	0.348	228	1.4
5	Back	29.1 ± 1.6	29.0 ± 1.3	0.823	487	3.0
6	Ribs	30.1 ± 1.3	30.3 ± 1.5	0.606	1,925	11.9
7	Breast	26.3 ± 2.1	25.6 ± 2.2	0.162	82	0.5
8	Fore flank	27.9 ± 2.2	28.0 ± 1.7	0.848	141	0.9
9	Costal cartilage	28.1 ± 2.1	28.0 ± 1.9	0.859	114	0.7
10	Subribs region	29.4 ± 1.8	29.6 ± 2.1	0.670	392	2.4
11	Loin	28.3 ± 1.9	28.9 ± 2.4	0.276	258	1.6
12	Hind flank	29.5 ± 1.5	30.0 ± 1.3	0.114	923	5.7
13	Abdomen	28.0 ± 1.2	28.1 ± 1.1	0.586	381	2.4
14	Coupling	29.6 ± 1.2	29.5 ± 1.0	0.588	160	1.0
15	Sheath	28.8 ± 2.6	28.0 ± 2.8	0.205	34	0.2
16	Groin	29.1 ± 3.1	29.2 ± 3.9	0.940	77	0.5
17	Dock	30.7 ± 3.2	29.4 ± 4.4	0.166	50	0.3
18	Shoulder	30.5 ± 4.2	30.9 ± 3.0	0.580	1,154	7.1
19	Arm	29.1 ± 4.1	29.7 ± 3.6	0.528	565	3.5
20	Point of shoulder	29.0 ± 4.3	29.9 ± 2.8	0.320	448	2.8
21	Elbow	27.7 ± 3.6	27.1 ± 3.8	0.467	220	1.4
22	Forearm	27.3 ± 5.0	27.2 ± 4.0	0.890	399	2.5
23	Knee	24.9 ± 4.2	24.6 ± 4.2	0.409	162	1.0
24	Cannon	21.8 ± 4.6	22.2 ± 4.0	0.625	255	1.6
25	Digit (phalanx)	26.9 ± 5.1	25.0 ± 4.0	0.082	261	1.6
26	Croup	29.1 ± 2.5	29.1 ± 2.0	0.966	453	2.8
27	Point of hip	28.5 ± 2.4	29.5 ± 2.1	0.060	45	0.3
28	Haunch	29.4 ± 2.7	29.9 ± 2.8	0.513	601	3.7
29	Point of buttock	29.8 ± 2.6	30.0 ± 2.3	0.728	42	0.3
30	Trochanter	28.7 ± 3.1	29.6 ± 2.7	0.243	48	0.3
31	Thigh	30.2 ± 3.7	30.5 ± 3.1	0.650	1,135	7.0
32	Stifle joint	28.0 ± 3.1	28.5 ± 3.4	0.569	326	2.0
33	Gaskin	26.4 ± 3.4	25.8 ± 3.4	0.517	601	3.7
34	Hock	25.1 ± 3.4	24.9 ± 5.0	0.377	160	1.0
35	Cannon	22.8 ± 5.5	23.1 ± 4.3	0.687	250	1.5
36	Digit (phalanx)	26.6 ± 4.6	27.1 ± 4.5	0.652	254	1.6
Total					16,183	100.0

of cannons, knee, hock and, breast and gaskin. The lower parts of the rear limbs were generally slightly warmer than the analogous parts of the front limbs, with the exception of the gaskin and forearm [9]. The areas around the digits were warmer than the areas around the cannons. Intermediate temperatures were recorded in the case of fore flank, costal cartilage, loin, stifle sheath and digits. The highest temperatures were identified in 20 places at the surface of the head, neck, trunk and upper parts of the limbs.

The above breakdown of areas at the surface of the horse's body at rest may be of diagnostic relevance. Their interrelations in respect of temperature are typical and any change in the above relations may be indicative of disease. It is important to compare the temperature of the given region with another region (temperature gradient) rather than the absolute numerical value of one of them. The surface area of each region and its percentage share of the entire surface area of the horse's side were calculated. Attention was paid to the fact that the largest clusters of maximum temperatures occurred over surfaces anatomically determined to be the largest, i.e. head, neck, shoulder, ribs and thigh. This indicated that they had a substantial share in the thermoregulation functions of the horse's organism.

After the show jumping competition, the range of the horses' body-surface temperatures was from 25.2 ℃ to 34.2 ℃ (Table 2). The lowest temperatures occurred at the knee and hock. The intermediate temperatures were

Table 2 Maximum body surface temperature of horses after competition.

No.	Region of interest (ROI)	Maximum temperature ($\overline{T}_{max} \pm S_T$ (℃))		Test L v. R *P* value
		Left side	Right side	
1	Head	30.9 ± 1.0	30.9 ± 1.2	0.829
2	Dorsal s. of neck	33.9 ± 1.2	33.3 ± 1.2	0.069
3	Ventral s. of neck	34.2 ± 1.5	33.8 ± 1.5	0.311
7	Breast	31.0 ± 1.2	31.0 ± 1.1	0.831
11	Loin	32.4 ± 1.5	32.1 ± 1.9	0.524
12	Hind flank	32.3 ± 1.7	33.1 ± 1.7	0.112
13	Abdomen	30.7 ± 1.9	31.1 ± 1.8	0.530
14	Coupling	32.0 ± 1.4	32.6 ± 1.3	0.066
15	Sheath	32.4 ± 1.0	32.3 ± 1.2	0.765
16	Groin	31.7 ± 1.8	31.6 ± 1.9	0.827
17	Dock	31.2 ± 1.6	31.6 ± 1.3	0.239
19	Arm	33.1 ± 0.6	33.1 ± 0.6	0.608
20	Point of shoulder	33.2 ± 1.7	32.9 ± 1.8	0.475
21	Elbow	32.4 ± 1.6	32.6 ± 1.7	0.627
22	Forearm	31.6 ± 1.3	31.7 ± 1.3	0.858
23	Knee	25.8 ± 1.7	25.2 ± 1.7	0.164
26	Croup	32.4 ± 1.7	31.5 ± 2.1	0.074
27	Point of hip	32.3 ± 2.2	31.9 ± 1.8	0.447
28	Haunch	32.9 ± 1.8	32.3 ± 1.9	0.272
29	Point of buttock	33.2 ± 1.3	33.1 ± 1.6	0.802
30	Trochanter	31.6 ± 1.4	31.7 ± 1.4	0.773
31	Thigh	33.1 ± 1.3	32.8 ± 1.8	0.580
32	Stifle joint	33.0 ± 1.8	32.6 ± 1.2	0.296
33	Gaskin	31.1 ± 1.8	31.3 ± 1.5	0.602
34	Hock	25.7 ± 1.0	25.9 ± 0.8	0.515

observed in the case of head, breast, abdomen, groin, forearm and gaskin, while the highest temperatures were recorded at the neck, arm, point of shoulder and thigh and another ten places. Similarly like before the competition, no statistically significant asymmetry in temperature between the right and left sides of the horses was found after the exercise [10, 11].

The greatest increase in maximum temperature following the competition was observed in the case of ventral s. of the neck, point of shoulder, elbow, croup, hip, haunch, stifle joint, gaskin (Table 3). This indicated that these regions played the greatest role in heat releasing from the horse's organism during an extreme work. However, no impact of effort on the increase in temperature was observed in the regions of the dock, knee and hock. On the other hand, the surface temperature of the head following the horse's exercise was slightly lower than before the competition. This could be explained by the functioning of the thermoregulatory centre in the hypothalamus, which controled the balance between heat-generating processes in the organism and its dissipation into its surroundings, on the basis of information sent by thermoreceptors.

The results presented in Table 3 may be interesting for horse riders and trainers in relation to the intensity of work of chosen regions of the body, in particular well-muscled regions. The following muscles had an

Table 3 Differences of body surface temperature * of horses, before and after competition.

No.	Region of interest (ROI)	T_{max} (℃) mean (min to max)		Mean (℃) difference	Two-tailed probability
		before competition	after competition		
1	Head	30.9 (30.5 to 31.7)	30.9 (30.6 to 31.2)	-0.02	$P = 0.6972$
2	Dorsal s. of neck	29.6 (29.1 to 30.4)	33.6 (33.3 to 33.9)	4.03	$P < 0.0001$
3	Ventral s. of neck	29.6 (28.9 to 29.9)	34.1 (33.8 to 34.5)	4.49	$P < 0.0001$
7	Breast	26.1 (25.5 to 27.9)	31.0 (30.7 to 31.3)	4.39	$P < 0.0001$
11	Loin	28.5 (28.1 to 29.6)	32.2 (31.8 to 32.7)	3.66	$P < 0.0001$
12	Hind flank	29.8 (28.9 to 30.2)	32.7 (32.3 to 33.2)	2.94	$P < 0.0001$
13	Abdomen	28.1 (27.4 to 29.3)	30.9 (30.4 to 31.4)	2.75	$P < 0.0001$
14	Coupling	29.5 (28.6 to 29.9)	32.3 (31.9 to 32.7)	2.81	$P < 0.0001$
15	Sheath	28.5 (28.1 to 29.3)	32.3 (32.0 to 32.6)	3.81	$P < 0.0001$
16	Groin	29.1 (28.6 to 29.1)	31.6 (31.1 to 32.1)	2.46	$P < 0.0001$
17	Dock	30.0 (29.2 to 30.9)	31.4 (31.0 to 31.8)	1.39	$P = 0.0026$
19	Arm	29.4 (28.8 to 30.9)	33.1 (32.9 to 33.3)	3.65	$P < 0.0001$
20	Point of shoulder	29.3 (28.0 to 30.2)	33.0 (32.6 to 33.5)	3.72	$P < 0.0001$
21	Elbow	27.4 (27.1 to 29.2)	32.5 (32.1 to 33.0)	4.96	$P < 0.0001$
22	Forearm	27.2 (25.9 to 28.3)	31.7 (31.3 to 32.0)	4.52	$P < 0.0001$
23	Knee	24.7 (23.4 to 26.0)	25.5 (25.1 to 26.0)	0.81	$P = 0.2722$
26	Croup	29.1 (28.4 to 30.4)	31.9 (31.4 to 32.4)	2.82	$P < 0.0001$
27	Point of hip	28.8 (28.3 to 29.4)	32.1 (31.5 to 32.6)	3.32	$P < 0.0001$
28	Haunch	29.6 (28.4 to 29.9)	32.6 (32.1 to 33.1)	3.04	$P < 0.0001$
29	Point of buttock	29.9 (29.3 to 30.7)	33.2 (32.8 to 33.6)	3.29	$P < 0.0001$
30	Point of hip	29.1 (28.4 to 29.8)	31.7 (31.3 to 32.0)	2.57	$P < 0.0001$
31	Thigh	30.4 (29.2 to 30.9)	33.0 (32.5 to 33.4)	2.60	$P < 0.0001$
32	Stifle joint	28.2 (27.2 to 30.1)	32.8 (32.4 to 33.2)	4.56	$P < 0.0001$
33	Gaskin	26.1 (25.3 to 28.2)	31.2 (30.8 to 31.6)	5.10	$P < 0.0001$
34	Hock	25.0 (23.9 to 26.9)	25.8 (25.4 to 26.1)	0.84	$P = 0.0747$

*arithmetic means and 95% confidence interval for the means.

influence on body-surface temperature above the knee: *Common digital extensor*, *Extensor carpi radialis* and *Ulnaris lateralis*, and above the hock: *Long digital extensor* and *Lateral digital extensor*. The surface temperature of the neck and trunk depends on the position of the following muscles: *Trapezius, Deltoideus, Long head, Lateral head, Cleidobrachialis, Subclavius, Latissimus dorsi, Extensor intercostal muscles, Extensor abdominal oblique* and *Internal intrecostal* [12].

The research regarding body surface temperature following an exercise were of no diagnostic value for veterinarians. It was proven in previous studies that "warming-up" certain parts of body surface masked an inflammation processes [13].

It must be emphasised that the horses being studied were taking part in competitions of varying difficulty levels (length of route, height and width of obstacles), hence their physical ability levels were not identical. Despite this fact, the differences in maximum temperature at the body-surface of individual horses before and after the competition were minor, as indicated by the standard variation levels, particularly around the region of the neck and trunk, which was indicative of the thermostability of these regions proven earlier [14].

However, the maximum temperatures of the limbs were characterised by a greater standard variation, which pointed to the thermolability of these regions. As an example, the temperatures of the surface before competition were thermolabile on the knee (ROIb 23) (Fig. 3) and thermostabile on the point of buttock (ROIb 29) (Fig. 4). After the show jumping, competition the temperature of point of buttock rose significantly, in comparison to knee. The knee lability decreased. Earlier publications proved the thermo-stability and thermo-instability of regions of the body in relation to variable micro-climatic conditions [14]. In this study, this was demonstrated in relation to horses subject to a workload.

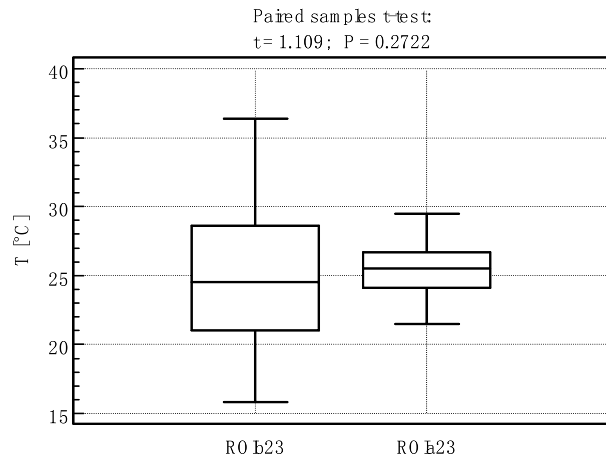

Fig. 3 Graphical characteristics of the surface temperature of knee (23) before (ROIb) and after (ROIa) exercise and the result of the student's t-distribution test (difference in temperature 0.8 ℃ statistically insignificant at a level of $P > 0.05$).

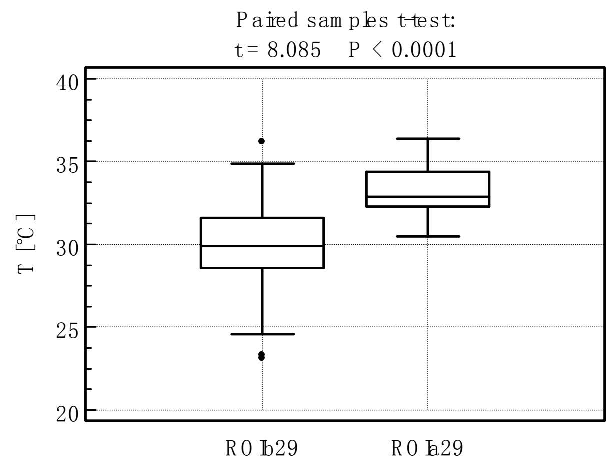

Fig. 4 Graphical characteristics of the surface temperature of point of buttock (29) before (ROIb) and after (ROIa) exercise and the result of the student's t-distribution test (difference in temperature 3.2 ℃ statistically significant at a level of $P < 0.0001$).

4. Conclusions

The study demonstrated the changes in body surface temperatures resulting from the exercise in particular body regions in horses. The choice of maximum temperatures is useful for characterising the distribution of temperatures for diagnostic purposes and for assessing the functioning of particular parts of the body during exercise. It is worth to emphasize that the interdependence of regions with maximum temperatures is significant in relation to the

musculature of particular areas. Horses are symmetrical as regards body-surface temperature measured from both sides, what means that any asymmetry discovered in this regard should be examined by a veterinarian. However the temperature level (℃) varies depending on individual characteristics and the environmental impact, the graphical distribution of body-surface temperatures is characteristic for all healthy horses regardless of their level of training. The knowledge of the body surface temperatures of horses in relation to the musculature of particular areas may be a useful tool in sports training planning.

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Optimum Insecticide Doses Determination of Seed Oil of Three Botanicals *Jatropha Curcas* L., *Helianthus Annus* L. and *Cocos Nucifera* L. against Maize Weevil *Sitophilus Zeamais* Mots.

Rachael Folake Ogunleye[1], Mary Omofolarin Ogunkoya[2] and Francis Olawale Abulude[2]

1. Department of Zoology, University of Ado-Ekiti, Ado Ekiti, Ekiti State PMB5363, Nigeria

2. Departement of General Studies, Federal College of Agriculture, Akure Ondo State 340001, Nigeria

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Abstract: The insecticidal effect of four dosage rates of three botanicals namely *Jatropha curcas, Heliathus annus* and *Cocos nucifera* was tested on the maize weevil *Sitophilus zeamais* Mots.. This is done for the purpose of finding a replacement for conventional insecticides which has been found to be harmful to man. The seed oil was applied topically at the rate of 0.1, 0.2, 0.3 and 0.4 mL per insect. There were a total of 20 insects per Petri-dish. There were four replicates per treatment. Insect mortality was recorded on 12 hourly basis for 48 hours. The results of insects treated with all dosage rates of *C. nucifera* showed a significantly higher mortality when compared with the control. In the case of *H. annus*, insect mortality ranged from 40-100, 70-100, 60-100 and 80-100% and for *J. curcas* the result ranged from 0.0-100, 40-100, 80-100 and 80-100% for rates of 0.1, 0.2, 0.3 and 0.4 mL, respectively, from 12 hrs to 48 hrs post application. The control experiment remained at 0% level throughout the period of the experiments.

Key words: Seed oil, botanicals insecticides, *Sitophilus zeamais*.

1. Introduction

One of the major constraints to crop production is the problem of insect pest infestation. Insect pests infest crops both in the field and also during storage. The storage insect pests are categorized into two groups. The first group is primary pests which include insects like *Callosobruchus maculatus*, *Sitophilus zeamais*, *Trogoderma granarium*, *Lasioderma sericorne* etc. They are capable of causing an economic damage if not controlled, within a period of 3-4 months of storage.

The entry and exit points of the primary pests create an avenue for secondary pests such as *Tribolium castanium*, *Tribolium confusum* etc to act. The bruises created by these pests also grant organisms such as bacteria and fungi access to the stored produce for further damage. Post-harvest crop losses have been estimated as 10-20% world wide but 25-40% for the tropics [1, 2]. Various control measures have been designed and practiced in order to control these notorious pests. Some of them are pest exclusion through legislation. However, in many developing countries, there is the problem of implementation and enforcement [3]. Another method is pest surveillance which involves the use of appropriate technology traps to detect and monitor insect pests of the various stages of growth. This method is only useful in large scale storage due to the expenses involved. Selection of improved crop variety is another method. These varieties have been found to succumb to the storage pests after a period of time [4]. Others are use of low temperature; this

Corresponding author: Rachael Folake Ogunleye, Ph.D., research field: applied entomology. E-mail: droluwaleye@yahoo.com.

involves storage of produce inside deep freezers [5]. For the farmers in West Africa however, this method of insect control is referred as controlled atmospheric storage. This involves flushing air-tight structures with carbon dioxide and nitrogen to give rapid and effective control [6, 7]. This may also be too expensive for resource poor farmers in the tropics.

The most effective insect pest control measure is the use of synthetic insecticides. Some of these insecticides include pirimiphos-methyl, fenitrothion, malathion, etc. The continued and intensive usage of these insecticides has produced some undesirable toxic effects on men handling them and also on non-target biotic components of the ecosystem. In order to solve this problem, researchers all over the world have to use more available and environmentally friendly botanicals. Several botanicals have been screened for insecticidal activities. These include *Zanthozylum zanthoxyloides*, *Nicotiana tabacum*, *Eugenia aromatic*, *Azadirachta indica, Capsicum*, *Dennetia tripetala* [8-11]. Efficacies of plant-derived oils have also been used to suppress the ever increasing pest population [12-14]. However, many of the botanicals have not been screened.

This paper aims at evaluating the insecticidal property of some indigenous plants seed oil for the control of *Sitophilus zeamais*.

2. Materials and Methods

2.1 Preparation of Seed Oil

The seeds of *J. curcas*, *H. annus* and *C. nucifera* were obtained within the vicinity of Federal College of Agriculture Akure, a town in southwest of Nigeria. They were then dehulled and air dried for 1 month. The dry seeds were ground in a Kenwood blender, and sieved with 40 mesh sieve and stored in screw-cap bottles and kept on the laboratory table under ambient conditions of temperature and pressure. The seed powder of the three plants were mixed properly with petroleum ether (b.pt 40-60%) then subjected to exhaustive extraction using the soxhlet extraction. After the extraction, the oils were left opened for 1 hour to allow any trace of petroleum vapour to escape before storage in containers.

2.2 Insect Culture

A pure culture of *S. zeamais* was maintained in the laboratory. Clean uninfested seeds of yellow maize were kept in kilner-jar like containers. Twenty randomly selected species of *S. zeamais* were introduced into the maize container and left on the laboratory table under ambient environmental conditions of 32 ℃ and 60% RH. The insects multiplied in the container within six weeks. The entire insects used for these experiments were taken from these containers.

2.3 Application of Seed Oil

Newly emerged adult insects (about one to three days old) were used for this experiment. The insect species were put in the refrigerator for 3 mins to immobilize them thereby preventing them from flying away. 20 adult insects which were randomly selected (without sexing) were placed in petri-dishes. Each of the prepared seed oil was applied to each of the insects separately at the application rates of 0.1, 0.2, 0.3, and 0.4 mL per insect per Petri-dish. Each of the experiments was replicated three times to make four replicates. For the control experiments, the insects in the replicated Petri-dishes were left untreated. The lids of all the Petri-dishes were perforated to give room for proper aeration. They were then left on the laboratory table and the percentage insect mortality was taken on 12 hourly bases for 48 hrs.

2.4 Statistical Analysis

All data collected were subjected to analysis of variance and the means were separated using Fisher's Least Significant Differences.

3. Results and Discussion

The result of the experiments involving different rates of application of *C. nucifera* oil on *S. zeamais* was recorded in Table 1. All the treatments produced

significant mortality effects on *S. zeamais* ($P \leq 0.05$) after 12 hrs of application when compared with control. For 0.1 and 0.2 mL rate, mortality ranged from 50-100% and 60-100% respectively, from 12 hrs to 48 hrs. Mortality of *Sitophilus* was as high as 70% for the application rates of 0.3 and 0.4 mL after 12 hrs and reached 100% after 24 hrs.

The result obtained in this study corroborated the work of G.P. Pandey et al. [15]. According to them, *C. nucifera* oil was effective in suppressing the population of some storage pests especially *C. maculatus*.

The mode of action, appropriate dosages and duration of efficacy of oils had been investigated by various workers on storage insect pests. Differences between crude and purified oil had been studied and crude oil had been found to be a better protectant [15-18].

Table 2 showed the mean percentage mortality of *S. zeamais* treated with different rates of application of the seed oil of *H. annus*. Like the result of the seed oil of *C. nucifera*, all the rates of application of *H. annus* oil produced a significant insect mortality (Using Fisher's Least Significant Difference) within the first 12 hrs post application. Mortality ranged from 40-100, 70-100, 60-100 and 80-100% for rates of 0.l, 0.2, 0.3, and 0.4 mL respectively from 12 hrs to 48 hrs after application.

The seed oil of *H. annus* was found to be a natural insecticide against storage pests particularly *A. obtectus* [19].

C.M. Ketker [20] observed that different oils of neem, coconut, and castor acted as surface protectants on green gram to check the pulse beetle and among them, neem oil was the best surface protectant. M. Ramzan [21] reported that cotton seed, sunflower, groundnut, soybean and mustard oils, when mixed with cowpea, completely suppressed adult emergence of *C. maculates*. E. Shaaya et al. [22] reported that edible oils are potential control agents against *S. zeamais* and can play an important role in stored-grain protection.

The result of the mean percentage mortality of *S. zeamais* treated with different rates of application of *Jatropha* oil is presented in Table 3. No mortality was observed in the control experiment throughout the period of the experiment. The least rate of application (0.1) however, produced 70% mortality after 36 hrs. In the case of 0.2 mL, mortality started from the first 12 hrs with 40% and gradually increased to 100% at the 48th hour after application. For high rates of 0.3 and 0.4 mL, the percentage mortality in both cases were as high as 80% after 12 hrs and reached 100% after 24 hrs.

J. curcas contains a purgative oil and a phytotoxin or (toxalbumin curcin) similar to ricin in Ricinus. Urchin - a phytotoxin (toxalbumin), found mainly in the seeds and also in the fruit and sap [23]. This genera also may contain hydrocyanic acid, there may also be an

Table 1 Mean percentage mortality of *Sitophilus zeamais* treated with different rates of application of *Cocos nucifera* (coconut oil).

Treatments (mL)	Mean Percentage of Mortality at different time (%)			
	12 hours	24 hours	36 hours	48 hours
Control	0.0 ± 00 a	0.0 ± 00 a	0.0 ± 00 a	0.0 ± 00 a
0.1	50 ± 00 bc	70 ± 7.10 bc	90 ± 00 bc	100 ± 00 b
0.2	60 ± 7.1 b	82.5 ± 10.90 bc	90 ± 00 bc	100 ± 00 b
0.3	70 ± 7.1 b	100 ± 00 b	100 ± 00 b	100 ± 00 b
0.4	70 ± 00 b	100 ± 00 b	100 ± 00 b	100 ± 00 b

Means followed by the same alphabets are not significantly different at 5% level using Fisher's Least Significant Difference. The same with the following tables.

Table 2 Mean percentage mortality of *Sitophilus zeamais* treated with different rates of application of sunflower oil.

Treatments (mL)	Mean Percentage of Mortality at different time (%)			
	12 hours	24 hours	36 hours	48 hours
Control	0.0 ± 00 a	0.0 ± 00 a	0.0 ± 00 a	0.0 ± 00 a
0.1	40 ± 00 e	60 ± 00 d	80 ± 00 bc	100 ± 00 b
0.2	70 ± 00 d	80 ± 00 c	100 ± 00 b	100 ± 00 b
0.3	60 ± 00 c	90 ± 00 b	100 ± 00 b	100 ± 00 b
0.4	80 ± 00 b	90 ± 00 b	100 ± 00 b	100 ± 00 b

Table 3 Mean percentage mortality of *Sitophilus zeamais* treatment with different rates of application of *Jatropha* oil.

Treatments (mL)	Mean Percentage of Mortality at different time (%)			
	12 hours	24 hours	36 hours	48 hours
Control	0.0 ± 00 a	0.0 ± 00 a	0.0 ± 00 a	0.0 ± 00 a
0.1	0.0 ± 00 a	0.0 ± 00 a	70 ± 7.1 bc	100 ± 00 b
0.2	40 ± 7.1 bc	50 ± 00 bc	70 ± 7.1 bc	100 ± 00 b
0.3	80 ± 7.1 b	100 ± 00 b	100 ± 00 b	100 ± 00 b
0.4	80 ± 00 b	100 ± 00 b	100 ± 00 b	100 ± 00 b

alkaloid, and a glycoside which produce cardiovascular and respiratory depression [24].

Characteristics of many members of the family *Euphorbiaceae Jatropha* plants contain several toxic compounds, including lectin, saponin, carcinogenic phorbol, and a trypsin inhibitor. Tetramethylpyrazine (TMPZ), an amide alkaloid, has been obtained from the stem of *J. podagrica* [25]. Atropine-like effects have also been reported following ingestion of *J. multifida* [26].

The results obtained from this study are a strong indication of the efficacy of these plant oils in suppressing the population of this notorious storage pest. In addition to the secondary plant metabolites found in this plants which could be toxic to the insects, it has also been reported that plant oils also have a suffocating effect on insect pests by way of preventing the easy passage of atmospheric air onto them [12].

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Ecological Study on Bacteria-Zooplankton Interaction in Domat Al-Gandal Lake, AL-Jouf Area, Kingdom of Saudi Arabia

Randa A. El-Bassat[1], Hussein E. Touliabah[2] and Najah I. Abdulwassi[3]

1. Department of Biology, Faculty of Science, Umm Al-Qura University, Makkah 21955, Kingdom of Saudi Arabia

2. Department of Biology, Rabigh College of Science and Arts, King Abdul-Aziz University, Rabigh 21911, Kingdom of Saudi Arabia

3. Department of Biology, College of Sciences, King Abdul-Aziz University, Jeddah 21589, Kingdom of Saudi Arabia

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Abstract: A year-long survey of some physical parameters (water temperature, dissolved oxygen, pH, turbidity), chemical parameters (Biological Oxygen Demand "BOD", Chemical Oxygen Demand "COD", nitrate, ammonia, orthophosphate) and some biological (zooplankton) and microbiological (total bacteria, indicator bacteria, pathogenic bacteria) components in Domat Al-Gandal Lake was conducted. Water samples were collected seasonally from spring 2004 to winter 2005. Four locations on the Lake were sampled in order to evaluate the condition of the Lake. To investigate the possible effect of zooplankton in controlling the presence of faecal indicator bacteria, a 24 hour experiment was carried out to examine this hypothesis as well as the grazing pressure of zooplankton on the bacterial community of the Lake. The results showed that variations in season temperature affected the zooplankton and density of bacteria in the Lake. Zooplankton was composed by Protozoa 75%, Rotifera 20.6%, Cladocera 3% and Copepoda 2%. The reduction rates for the tested faecal pollution indicators in presence of zooplankton predators were remarkable (up to 99%). In conclusion zooplankton, especially protozoa, was found to play an important role as biological control against bacterial indicators of faecal pollution.

Key words: Bacteria-zooplankton interaction, grazing, water quality, pathogens.

1. Introduction

Dumat al-Jundal is a ruined ancient city located in North Western Saudi Arabia in the Al Jouf province. It is the oldest city in Aljouf Reagon. The name Dumat Al-Gandal means literally "Dumah of the Stone", since this was the territory of Dumah one of the twelve sons of Ishmael.

Domat Al-Gandal lies at about 880 km northwest of Riyadh, with a surface area of one million km^2 (Fig. 1). The lake lies at about 500 km below sea level, with 2 km length and 1 km width. It extends from 39°54′499″E to 29°48′590″N, the Lake receives the agriculture drainage from the surrounding area, and it also considers the collecting place rains falling on the nearby mountains.

Water pollution problems are increasing over time. The major source of Domat Al-Gandal pollution is the disposal of untreated domestic sewage into the lake. The level of sanitation service, especially in rural areas (7% at most), is low. Excessive use of fertilizers and pesticides is another major source of water pollution.

Zooplanktons play a pivotal role in aquatic food webs because they are important food for fish and invertebrate predators and they graze heavily on algae, bacteria, protozoa, and other invertebrates. Zooplankton communities are highly sensitive to environmental changes. As a result, changes in their

Corresponding author: Randa A. El-Bassat, Ph.D., associate professor, research field: invertebrate ecology. E-mail: relbassat@yahoo.ca.

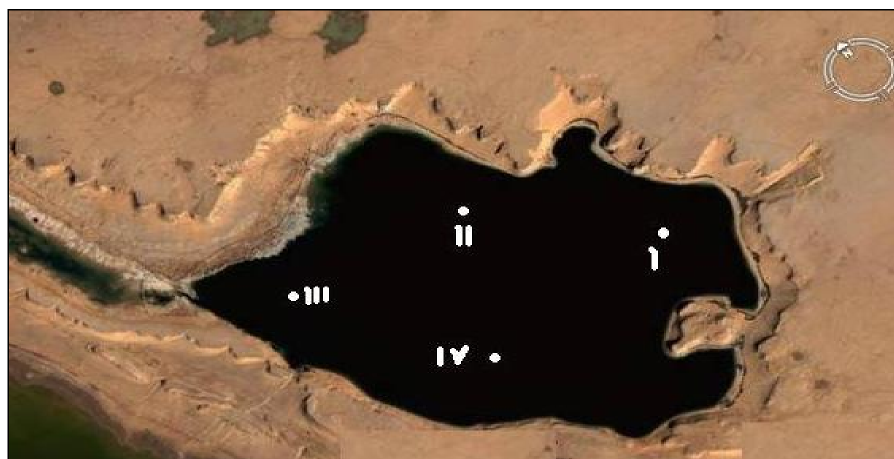

Fig. 1 Satellite photo of Domat Al-Gandal Lake in Al-Jouf Area.

abundance, species diversity, or community composition might provide important indications of environmental changes or disturbance [1]. Zooplankton communities respond to wide variety of disturbance including nutrient loading [2, 3], acidification [4], contaminants [5], water temperature, light, pH, oxygen, salinity, toxic and food availability [1].

Bacteria are microscopic, single-celled organisms that can be found in virtually any environment. Indicators bacteria in a waterway come from many sources e.g., animal droppings, faulty or leaking septic or sewage systems, combined sewage overflows, storm water runoff, boat sanitary wastes and disturbed sediments. Contaminated water is an important vehicle for the spread of water-borne diseases [6]. The use of normal intestinal organisms as indicators of faecal pollution rather than the pathogens themselves is a universally accepted principle for monitoring and assessing microbial safety of water supplies. Organisms commonly used as bacterial indicators for faecal pollution are the coliform group including faecal coliforms and faecal streptococci [7-9]. The use of normal intestinal organisms as indicators of faecal pollution rather than the pathogens themselves is a universally accepted principle for monitoring and assessing microbial safety of water supplies.

In aquatic ecosystems, the role of bacteria and picoplankton as food resources for different planktonic organisms has been long recognized [10], and different groups of grazers including flagellates, ciliates, rotifers and crustaceans may compete for these resources [11]. Metazoans and protozoans graze on bacteria and picoplankton, and the relative importance of each group on the bacteria grazing losses has been determined in natural environments [12-14].

The present study aimed to study the seasonal fluctuations in different zooplankton groups in Domat Al-Gandal Lake, and the effect of organic pollution. Examination of the planktonic bacteria, its seasonal variations along the investigated area as and survey of the faecal indicators bacteria at the selected stations were of our targets during the present study.

During the present study, the authors assumed that zooplankton as predators of bacteria might be able to biologically control the presence of faecal indicators bacteria, so an experimental work was carried out in the laboratory to examine the grazing pressure of microzooplankton on bacterioaplankton.

2. Materials and Methods

2.1 Field Measurements

2.1.1 Physical and Chemical Parameters

The chosen points were subjected to seasonal sampling program from spring 2004, up to winter 2005. Water temperature and dissolved oxygen (D.O.) were measured in the field at depth of 50-100 cm with D.O. Meter (WTW) model Oxi 315i. pH values were

measured using pH meter (INO Lab. WTW, pH electrode Sentix 41). The turbidity was measured using Turbidity Meter (Turbidity Thermo Orion 215.2). Samples for chemical analysis were collected in specific bottles from each point and preserved in ice tanks until transported to Lab. for analysis according to Ref. [15].

2.1.2 Biological Parameters

For protozoan samples one liter of lake water was collected with a water sampler from the subsurface layer of each station. The collected samples were preserved in 2% formalin solution. Organisms were identified to the highest possible taxonomic level (genus or species) using Refs. [16-19].

For zooplankton samples, fifty liters of water were collected from the subsurface layer. Samples were filtered through standard plankton net with 50 µm meshes. The collected samples were preserved in 4% formalin solution. Organisms were identified using Refs. [16, 20].

2.1.3 Microbiological Parameters

Water samples were collected in sterilized 750 mL bottles. Total bacterial count (the spread plate method), total coliform, faecal coliform and faecal streptococci were examined using the membrane filter method according to slandered method [15].

2.2 Grazing Experiment

Forty liters of Lake Water were collected with a plastic container from the subsurface layer. The water was divided into two groups, A and B. Group A was filtered using plankton net with pore size 5 µm and used to fill triple series of flasks with one liter each, so group A is a grazers free . On the other hand, group B was filtered through 55 µm plankton net, and used to fill triple series of flasks with one liter each, representing the grazers group. The two groups of samples were incubated at room temperature.

The experimental design was used to examine the numerical response of bacteria to plankton grazers at zero time, after 2, 4, 6, and 24 hours.

2.2.1 Bacterial Examination

Bacteriological media used were prepared according to Ref. [21] using the membrane filter technique with membrane filter pore size 0.45 µm (chm, Cellulose nitrate membrane). The procedures followed the detection of different bacteriological parameters were described in the standard methods for examination of water and waste water [15].

2.2.2 Protozoa and Zooplankton Examination

Samples at each time interval were fixed by adding 1 mL of 4% formalin and concentrated to about 25 mL. 1 mL from the sub-samples was transferred to a Rafter Cell and examined under a compound binocular microscope.

2.2.3 Statistical Analysis

Canonical corresponding analysis (CCA) was carried out using MVSP ver. 3.1 Program. Multiple regressions were done by Statistica program ver. 5.2. The previous analysis was performed on different plankton groups and physico-chemical parameters in order to determine the possible relations between them during the present study.

3. Results

3.1 Physical Conditions in the Area of Investigation

The minimum value of water temperature reached 16.1 ℃ during winter at the first station and increased to 44.5 ℃ during summer at station (III). During the period of investigation the dissolved oxygen values ranged between a minimum value of 2.8 mg/L during summer recorded at station (II) and a maximum value of 10.5 mg/L observed at station (II) during autumn. Values of pH of the study area lay in the alkaline side, with a small range of variation. The pH values ranged between 7.6 recorded at station (III) in spring and 8.8 recorded during autumn at station I and III. Water turbidity widely varied between the four stations of the investigated area. The minimum value was 2.5 Nephelometric Turbidity Unit (NTU) recorded at station III in winter, while the maximum was 12.1 NTU at station (I) during spring. The seasonal average

values of physical and chemical parameters are shown in Table 1.

3.2 Biological Oxygen Demand (BOD, mg/L)

The BOD values reached its maximum value 7.0 mg/L at station (I) in autumn. The winter values of BOD were 0.0 mg/L at all stations. During the period of investigation, the highest values were recorded during spring, while the lowest values were observed in the winter season.

3.3 Chemical Oxygen Demand (COD, mg/L)

The average values of COD were wide different along area of study. At station (II) the minimum value recorded 5 mg/L during summer season, while the maximum value was 39 mg/L recorded at station (II) of in winter.

3.4 Nutrients

The nitrate during the present study ranged between value of 0.24 mg/L during summer at station (I) and 3.29 mg/L at station III of during winter. Ammonia recorded a maximum value of 0.127 mg/L in spring at station (I), while orthophosphate ranged between 0.010 mg/L during spring at station (II) and 0.120 mg/L during summer at station (II).

3.5 Total Zooplankton Community

During the present study, zooplankton population in the investigated area was represented by Protozoa 75%, Rotifera 20.6%, Cladocera 3% and Copepoda 2%, respectively. Along the investigated area, zooplankton standing crop showed three major peaks, the first peak was 2,046 organism/L recorded at station (II) during spring. Secondly, a density of 1,964 organism/L was observed at station III during the winter season, thirdly 1,749 organism/L was recorded at station (III) in autumn. The minimum zooplankton abundance was 437 organism/L at station (I) of during the summer season. The seasonal averages of total zooplankton (Fig. 2) showed that lowest population densities along the investigated area were recorded in summer.

3.6 Bacterial Indicators

Concerning the seasonal average differences of Total

Table 1 Seasonal average values of some physical and chemical parameters of the Domat Al-Gandal Lake selected stations.

	Temperature /℃	Turbidity /NTU	pH	D.O_2 /mg·L^{-1}	BOD /mg·L^{-1}	COD /mg·L^{-1}	NO_3 /mg·L^{-1}	PO_4 /mg·L^{-1}
Station I	21.91	5.87	8.35	7.83	1.75	19.50	1.16	0.02
Station II	21.99	5.23	8.49	8.59	2.58	25.00	1.10	0.03
Station III	22.03	6.23	8.49	9.34	2.67	25.50	1.25	0.03
Station IV	23.60	6.38	8.37	9.63	1.83	23.58	1.30	0.02

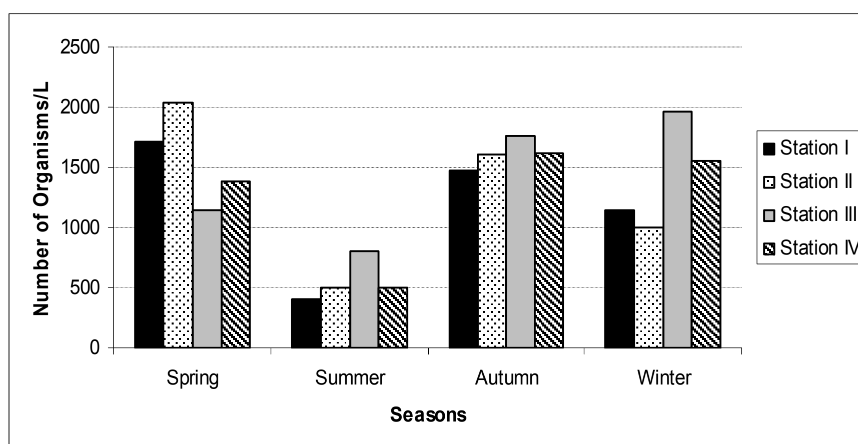

Fig. 2 Average densities of zooplankton population recorded in Domat Al-Gandal Lake (No. of organisms·L^{-1}).

Coliform, a peak with a value of 4,499.17 cfu/100mL was recorded at station III, while the lowest average was 765.83 cfu/100mL observed at station I. The Faecal Coliform the highest average was 495.83 cfu/100mL recorded at station IV, while the minimum average was 125.17 cfu/100mL observed at site II. The Faecal Streptococci maximum seasonal average was 88.92 cfu/100mL recorded at station III, while the minimum seasonal average was 23.50 cfu/100mL recorded at station II (Table 2).

3.7 Statistical Analysis

The canonical correspondence analysis (CCA) of the different zooplankton groups against some water quality parameters and bacterial community was shown in Fig. 3, in this kind of analysis the length of vector of a given variable on the CCA plots indicated the strength of the relationship between that variable and zooplankton groups. During the present study, CCA plot revealed that total Rotifera & total Protozoa were negatively correlated with DO, COD, NO_3 and pH, while total bacterial count at 22 ℃ had positive correlation with PO_4. Total bacterial count at 37 ℃ had positive relation with temperature, while *Faecal coliform* had a positive correlation with turbidity. On the other hand, *Faecal streptococci* had strong positive correlation with COD & NO_3.

3.8 Grazing Experiment

Water samples during the grazing experiment were divided into 2 groups, Group (A) and Group (B). In Group A, where all zooplankton grazers were removed by 5 μ screen, the bacterial count increased gradually from zero time to the end of the experiment

Table 2 Average counts of total coliform, faecal coliform and faecal streptococci (cfu/100mL) at the investigated area.

	Total coliform	Faecal coliform	Faecal streptococci
Station I	765.83	162.08	24.00
Station II	810.83	125.17	23.50
Station III	4,499.17	340.25	88.92
Station IV	1,710.00	495.83	41.33

(24 h), showing the normal growth curve. The total coliform count started at zero time with 1,700 cfu/100mL increased to 8,300 cfu/100mL by the end of the experiment (after 24 h). The faecal coliform increased in a similar way to total coliform, starting at zero time with 460 cfu/100mL, and increased to 960 cfu/100mL after 24 h. In case of faecal streptococci the count at zero time was 310 cfu/100mL increased to 500 cfu/100mL by the end of the experiment.

In Group B grazers more than 55 μ were removed and only the small grazers (microzooplankton) and bacteria were observed. The count of bacterial indicators started to decrease gradually from zero time to the end of the experiment. The total coliform started with 2,400 cfu/100mL and declined to 1,400 cfu/100mL after 24 h. The faecal coliform recorded 465 cfu/100mL at zero time and declined to 160 cfu/100mL after 24 h. On the other hand, the faecal streptococci were 390 cfu/100mL at zero time and reduced to 120 cfu/100mL after 24 h (Table 3).

The Protozoa and Rotifera were the two main grazers in the experiment. Protozoa formed 90% of total zooplankton crop, while Rotifera constituted 10% of total zooplankton. Protozoa was dominated in the experiment by six genera *Vorticella, Epistylis, Strombidium, Tintinnidium, Halteria*, and *Difflugia*. Rotifera was dominated by four genera *Trichocerca, Polyarthra, Philodina*, and *Collotheca*.

3.9 Statistical Analysis

The correlation regression analysis for the bacterial counts against time in group A revealed a highly significant positive relationship ($r = 0.95$, $P < 0.0001$).

In group B: The regression analysis of various protozoan and rotiferan genera versus the different bacterial groups (TC, FC and FS) revealed the following relations:

The protozoan genus *Vorticella sp.* had the most effective grazing pressure on the three bacterial groups. Highly significant negative relations were established

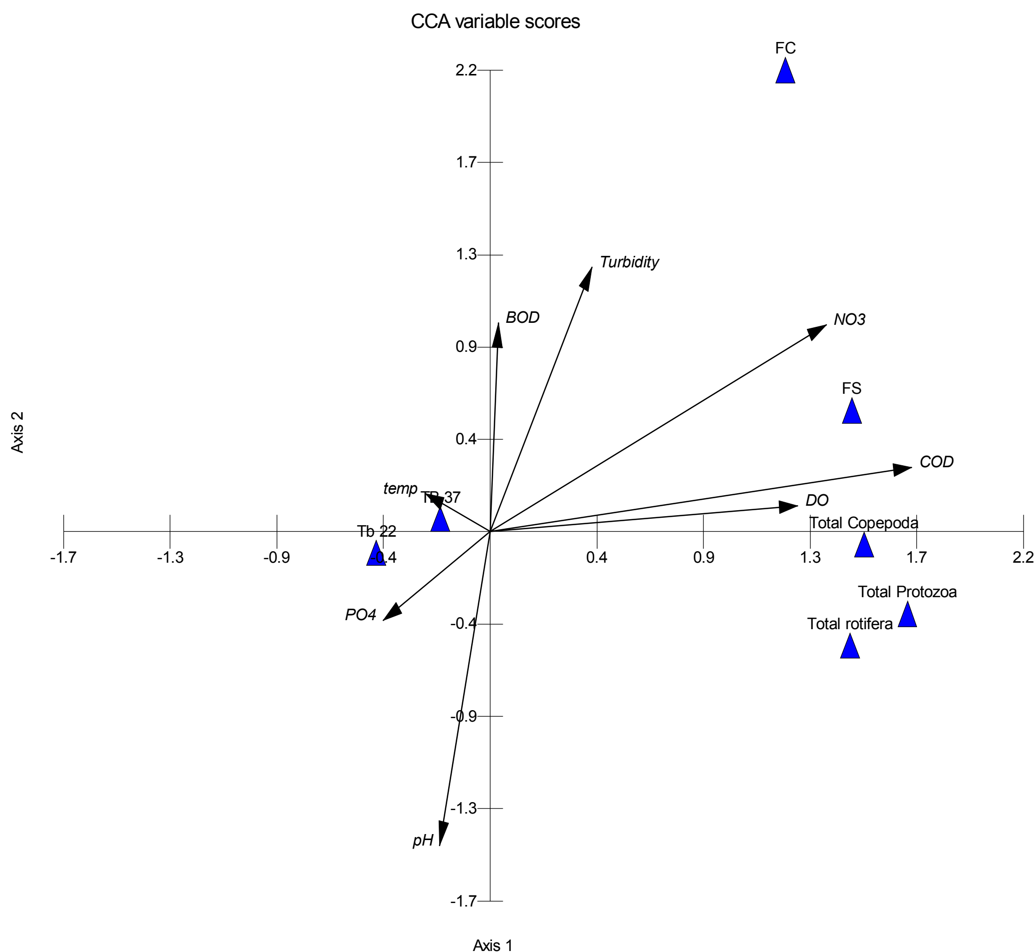

Fig. 3 CCA groups-environmental ordination on axis 1 and 2 biplot.

Table 3 Total counts of bacterial indicators and zooplankton grazers during the grazing experiment.

			0 hour	2 hours	4 hours	6 hours	24 hours
Group A	Grazers		Removed by filtration over 5 μ screen				
	Bacteria (cfu/100mL)	TC	1,700	2,300	3,500	6,000	8,300
		FC	460	510	520	800	960
		FS	310	380	440	430	500
Group B	Grazers (Org./L)	Protozoa	1,601.7	2,789.6	1,358.2	2,644.2	5,293.7
		Rotifera	255.7	222.9	423.5	157.3	307.6
		Zooplankton	1,857.4	3,012.5	1,781.7	2,801.5	5,601.3
	Bacteria (cfu/100mL)	TC	2,400	2,350	2,200	2,000	1,400
		FC	465	420	393	357	160
		FS	390	340	310	260	120

between *Vorticella sp*, total coliforms, Faecal coliforms and Faecal streptococci (r = -0.95, -0.92 and -0.94 respectively; $P < 0.0001$). The second protozoan genus with effective grazing pressure was *Strombidium*, which recorded highly significant negative relations with TC, FC and FS (r = -0.94, -0.92 and -0.9 respectively; $P < 0.0001$).

Among the rotifer genera, *Philodina sp*. was the most affecting species on the count of different bacterial groups during the experiment. *Philodina sp.*

attained a higher grazing pressure on the faecal coliform bacteria with significant indirect relation (r = -0.9, $P = 0.001$) more than on total coliforms and faecal streptococci (r = -0.87 and -0.85 respectively).

The experiment duration also attained a highly significant inverse relationship with the bacterial count in presence of the grazers (r = -0.97, $P < 0.0001$), while a significant positive relation was recorded between the abundance of both protozoan and rotifer grazers and time of experiment (r = 0.9 and 0.87, respectively, $P < 0.001$).

4. Discussion

In the present investigation, the decrease in oxygen concentration at the first station might be due to oxygen consumption in decomposition of organic matter by bacteria which recorded the highest count at this station [22].

The low value of turbidity recorded during winter can be attributed to cladocera which recorded high crop during this season. In this connection, clear-water phase (CWP) in deep Lake Constance (Europe) was attributed to strong grazing either by a daphnid-dominated zooplankton community or by a diverse assemblage consisting of micro- and meso-zooplankton [23]. During the present study a positive significant relation was reported between turbidity and faecal coliform, indicating the bad effect of turbidity on water quality.

The high BOD values during winter can be explained by the fact that microorganisms, mainly bacteria, use oxygen to break down the organic material and cause the biochemical oxygen demand (BOD) to increase within the system. A high demand lowers the availability of dissolved oxygen in the water due to the microbial activity [24].

The highest COD value recorded at station (I) during autumn might be due to low water flow which reflected the high load of organic matter at this station.

Ammonia concentration in unpolluted waters usually was found to be less than 0.2 mg/L as Nitrogen [25]. Higher concentration of ammonia could be attributed to organic pollution resulted from domestic sewage. This fact confirms the organic pollution found at Domat Al-Gandal Lake.

The zooplankton community in the area of investigation was dominated by Protozoa and Rotifera. The controlling factor of zooplankton community was probably the food availability (bacteria) associated with dissolved oxygen and temperatures in addition to seasonal variation [26, 27].

Summer season recorded the lowest zooplankton crop in comparison with other seasons during the investigation period. This result was similar with Refs. [28, 29] in Ogochi Reservoir, Tokyo. This decline might be attributed to fish predation which often regulated the biomass of zooplankton [30, 31]. The spring peak of protozoa in the present study agreed with R.A. El-Bassat [28], who recorded the protozoan flourishing in spring and autumn.

Concerning regional average variation, station (II) recorded the maximum average density of protozoa; this may be attributed to organic pollution. The preference of protozoan organisms to the area with high organic matter was reported before by Refs. [28, 32] and was supported statistically during the present study by CCA and multiple regression with significant negative relations with both DO and COD ($P < 0.01$).

Rotifers recorded the highest crop during autumn, while recorded the lowest crop during summer. The low rotifer abundance during summer season might be due to lower values of DO due to increase in water temperature. During the present study CCA results (Fig. 3) revealed significant negative relations between rotifers and DO, COD as well as NO_3. Flourishing of rotifers during autumn might be attributed to suitable water temperature low concentration of algae. This result agreed with observations of Refs. [33-35].

Spring recorded the maximum average count for total coliform at station (II) during the period of study.

This peak may be attributed to suitable water temperature during this season and presence of organic matter. On the other hand, the minimum average count recorded during autumn can be attributed to high zooplankton crop (protozoa and rotifers).

During the investigation period, a grazing experiment was done to evaluate the effect of zooplankton grazing activity on the indicator bacteria (total coliform, faecal coliform and faecal streptococci) during 24 hours. The reason for choosing zooplankton organisms less than 55 μ was according to a previous study carried out by R.A. El-Bassat et al. [36]. During their study, they found that these sizes of zooplankton grazers were more effective than the older ones. The most efficient group among zooplankton grazers varied from water body to another according to several biotic and abiotic parameters. For example, In River Lorie (France), it was observed that ciliates communities were the most abundant grazers [37, 38].

During the present experiment, in group B total coliform, *faecal coliform* and *faecal streptococc*i started to decline gradually from zero time until the end of the experiment as a result of the grazing pressure.

The zooplankton grazers were protozoans and rotifers with obvious domination of Protozoa. This result agreed with R.A. El-Bassat et al. [36] who recorded protozoa as dominant grazers in their grazing experiment.

The present study offered a possible control measure for the faecal pollution problems in water bodies. The grazing experiment confirmed the positive effect of protozoa and rotifers in the reduction of bacterial abundance. This result agreed with R.A. El-Bassat and S.A. Abdallah [39] who reported that zooplankton grazing was very important in regulating bacterial abundance and production in natural systems. Protozoa, especially heterotrophic nano-flagellates and ciliates (with the ability to graze on a variety of bacterial species), were usually the major consumers of bacteria in water environments [13]. They also added that Protozoa might influence the bacterial community structure.

From the results of the grazing experiment, it was clear that protozoa, especially ciliated species, played a very important role in grazing bacteria followed by Rotifera. This finding agreed with R.A. El-Bassat and S.A. Abdallah [39] who reported that protozoa was followed by Rotifera as the grazers in their grazing experiment. Also, the result agreed with several studies which highlighted the importance of heterotrophic flagellates and ciliates in the predation and control of picoplanktonic algae and bacteria [40-44].

The effective reduction of all faecal pollution indicators by zooplankton grazers during the present study suggests a recommendation of using this natural way for getting rid of these indicators from aquatic systems subjected to faecal pollution. In conclusion, zooplankton especially protozoa can play a very important role as biological control against bacterial indicators of faecal pollution of River Nile. Thus, they might be used in pre-treatment in water purification plants.

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Laboratory Assessment of Diatomaceous Earth against Several Stored-Grain Pests of Sorghum

Zlatko Korunic[1], Vlatka Rozman[2] and Anita Liska[2]

1. Diatom Research and Consulting Inc., Toronto, ON, M1W 1J2, Canada

2. Faculty of Agriculture, University of J. J. Strossmayer in Osijek, Trg Sv. Trojstva Osijek 3, 31000, Croatia

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Abstract: A wide range of insect pests attacks stored sorghum grains and the significant damage in grain weight loss and negative impact on the nutritional values of sorghum are caused with the activities of these pests. The insecticides, especially from organophosphate group (OP), are still used to prevent damage of sorghum grains during storage period. One of the possible replacements for OP and other synthetic insecticides is a natural and safe diatomaceous earth (DE). The primary objectives of this study was conducting the laboratory experiments with enhanced DE Protect-It on *Sitophilus oryzae* (L.), *Rhyzopertha dominica* (Fab.) and *Tribolium castaneum* (Herbst.) to find out the effective concentrations and to determine the effect of applicable concentration of DE on bulk density (test weight) of sorghum grains. The effect of Protect-it on bulk density was measured at 1,000 ppm. After the exposure of *S. oryzae*, *R. dominica* and *T. castaneum* to treated grains during 13 days, lethal dosis that causes 90% mortality (LD_{90}) were 811 ppm, 1,102 ppm and 1,244 ppm, respectively. After exposure of 8 days the concentration of 1,000 ppm generated the mortality of *S. oryzae* 100% and for *R. dominica* and *T. castaneum* for over 99%. The same concentration had a minimal effect on the reduction of sorghum bulk density (for 1.95% only).

Key words: Sorghum grains, storage, insects, diatomaceous earth, control, bulk density.

1. Introduction

A wide range of insect pests attacks stored sorghum grains (*Sorghum bicolor* L. Moench), especially in the arid and semi arid tropics. The most frequent species are *Rhyzopertha dominica* (Fab.), *Sitophilus oryzae* (L.), *Tribolium castaneum* (Herbst.), *Sitotroga cerealella* (Oliv.), *Ephestia cautella* (Walk.), *Plodia interpunctella* (Hubn.) and *Corcyra cephalonica* (Staint.). Significant damage is caused with the activities of these pests. The loss depends on insect species and cultivar and may be up to 66% [1]. Besides the loss in weight, higher populations affect the nutritional values of sorghum grain [2, 3]. Stored-grain insects usually infest sorghum in granaries soon after harvest unless control measures, mostly application of organophosphates insecticides are applied. Many conventional pesticides can adversely affect the environment, therefore requirements for safer means of pest management have become crucial. Chemical inputs are seen to be a primary cause of food contamination and environmental pollution arising from agriculture [4]. According to Kansas Agriculture Statistics in Kansas during 1998/99 approximately 13% of stored sorghum was treated with organophosphate malathion or chlorpyrifos methyl with direct spraying, top dressing and dusting [5]. The research today is focused on the replacement of organophosphate and other insecticides with good management practices integrated with minimum use of insecticides. Natural, safe to mammalians, diatomaceous earth (DE) is increasingly used as a part of integrated pest management (IPM) for stored grains

Corresponding author: Zlatko Korunic, Ph.D., research fields: stored product protection, pest control, inert dusts especially diatomaceous earth, development of natural, safe and low toxicity insecticides. E-mail: zkorunic@rogers.com.

[6, 7]. Depending on the product, DE provides long term control of infestation and can be comparable in cost to other control methods [6-8]. Until now there had been only few published papers to study the efficacy of DE for pests of stored sorghum [9-11].

The primary objectives of this study was to conduct laboratory experiments with enhanced DE Protect-It on three stored grain insect pests: *Sitophilus oryzae* (L.), *Rhyzopertha dominica* (Fab.) and *Tribolium castaneum* (Herbst.) to find out the effective concentrations and to determine the effect of applicable concentration of DE on bulk density (test weight) of sorghum grain.

2. Materials and Methods

The adult insects of the species *S. oryzae*–rice weevil, *R. dominica*–lesser grain borer and *T. castaneum*–red flour beetle were used in experiments. Specimens of mixed age were taken from cultures maintained at 30 ± 1 ℃, 70 ± 5% r.h. The cultures have been reared in the laboratory more than three years.

The test sorghum was produced in the USA (Kansas). The samples were not commercially cleaned (2.5% dockage) and had a moisture content of 13.0%. Insects were placed on treated grain, 50 insects/jar, 200 g of grain/jar. Each jar comprised one replicate and there were three replicates per treatment. For each treatment Protect-It was added to sorghum in a 1-litre glass jar and was shaken by hand for 1 minute to ensure thorough mixing. The study was comprised of two comparative tests. Test I included all three species at concentrations of 0, 400, 600, 800 and 1,000 parts per million (ppm), and test II at 0 and 1,000 ppm.

During the exposure period the test jars were kept at 30 ± 1 ℃, 70 ± 5% r.h. The assessments of the bioassays were conducted according to the following schedule: Test I, 5 and 13 days; Test II, 4 and 8 days. After the final assessment for each test, all the remaining living adults were removed and the grain was held at 30 ± 1 ℃, 70 ± 5% r.h for another 40 days. Bioassays were then done to estimate the number of offspring generated.

In order to determine the effects of Protect-It on bulk density, 3-500 grams of sorghum was mixed with 1,000 ppm of Protect-It and shaken in a jar for 1 minute. The test weight was measured using procedures outlined in the Canadian Grain Commission, Grain Grading Guide. Both treated and untreated grain was tested to establish the effect of Protect-It on test weight.

Statistical analysis: The LD_{50} (lethal dose for 50% of the population) and the LD_{90} (lethal dose for 90% of the population) was estimated using probit analysis (Test I) and effectiveness was analyzed by One Way ANOVA, $P = 0.05$ (Test II) [12].

3. Results

The LD_{90} values for Protect-It on sorghum (Table 1) were as follows: *S. oryzae* at 1,125 ppm (5 days) and 811 ppm (13 days); *R. dominica* at 1,414 ppm (5 days) and 1,147 ppm (13 days); *T. castaneum* at 1,573 ppm (5 days) and 1,244 (13 days).

For Test II (concentrations 0 and 1,000 ppm), there was 100% mortality after 8 days for *S. oryzae*, and over 90% mortality for *R. dominica* and *T. castaneum* (Table 2). In Test I progeny was controlled (LD_{90}) at 797 ppm for *S. oryzae*, at 1,144 for *R. dominica* and at 1,059 ppm for *T. castaneum*. The concentration of 1,000 ppm reduced the progeny of *T. castaneum* for 100% and *R. dominica* and *S. oryzae* for more than 99% (Table 2).

The test weight of untreated sorghum was 76.74 kg/hl. After mixing with 1,000 ppm of Protect-It, test weight was reduced by 1.5 kg/hl, or by 1.95% (Table 3).

4. Discussion

The species tested in this study are among the most difficult to control with contact insecticides, such as DE. The *R. dominica* is difficult to control due to its behavior. If they have not succumbed to the DE by the time they have bored their way into the kernel, they are no longer exposed and may be able to recover. The *T. castaneum* is also very tolerant to DE, but for reasons more physiological than behavioral. *S. oryzae* adults

Table 1 The LD_{50} and LD_{90} (90% confidence intervals) for Protect-It on 13.0% m.c. sorghum for three stored-product insect pests.

Insect species	Duration (days)	LD_{50} (ppm) (90% CI)	LD_{90} (ppm) (90% CI)	90% Reduction of offspring (ppm)
Sitophilus oryzae	5	630 (519 - 764)	1,125 (777 - 1,729)	797 (670 - 975)
	13	533 (450 - 630)	811 (647 - 1,054)	
Rhyzopertha dominica	5	842 (694 - 1,023)	1,414 (901 - 2,385)	1,144 (910 - 1,492)
	13	711 (610 - 829)	1,102 (831 - 1,531)	
Tribolium castaneum	5	1,018 (801 - 1,295)	1,573 (906 - 2,987)	1,069 (825 - 1,444)
	13	824 (706 - 962)	1,244 (897 - 1,819)	

Table 2 The efficacy of Protect-It at 1000 ppm against three stored-grain insects.

Insect species	Duration (days)	Mortality (%) ± std. dev.	Reduction of offspring (%)
Sitophilus oryzae	4	92.66 ± 2.30 b	99.2
	8	100.00 ± 0.00 a	
Rhyzopertha dominica	4	86.00 ± 4.00 b	99.7
	8	93.33 ± 2.30 b	
Tribolium castaneum	4	90.66 ± 3.05 b	100.0
	8	93.33 ± 3.05 b	

* means followed by different letters are significantly different ($P = 0.05$). The same with Table 3.

Table 3 The effect of 1,000 ppm of Protect-It on sorghum test weight (bulk density).

Test weight (kg/hl)			
Untreated	Treated	Reduction	
76.74 a	75.24 b	1.5 kg/hl	1.95%

are more sensitive to DE, but again the behavior of the insect can limit exposure to the insecticide. *S. oryzae* females lay eggs inside of the kernels and larvae and pupa develop inside of the kernel. This makes it more difficult to control *S. oryzae* progeny [13]. Certainly, the faster one can achieve mortality of infesting insects, the less likely it is that they will establish within the given commodity. However, from an economic perspective, it is important to use only enough products to provide the assurance that insect populations will not develop. Diatomaceous earth applied to a commodity should remain effective long after application as long as there is no appreciable increase in the moisture content of the commodity [8].

LaHue [9] compared the effectiveness of malathion and synergized pyrethrum emulsions and the diatomaceous earth product, Perma Guard, on sorghum in controlling existing insect infestations and preventing the establishment of other infestations during a continuous exposure to free-moving insect populations released in the storage area. Perma Guard was applied at rate of 4.17 pounds per ton (2,085 ppm) on sorghum with a moisture content of 13.43%. Perma Guard was effective against rice weevil only during the first month after treatment and had no adverse effect on the confused flour beetle *(Tribolium confusum)* in any of the tests. The repellency of DE lessened as the grain became more heavily infested and damaged during storage.

Stathers et al. [10] tested two commercially available DE products, Protect-It and Dryacide, against the major post-harvest insect pests of grains and pulses. On-farm field trials in Zimbabwe showed that both inert dusts gave significant protection against insect damage when admixed with farm stored sorghum for periods of 40 weeks. During the same storage period, untreated grain was heavily damaged. Stathers et al. [11] recommended the use of DE Protect-it at the concentration of 0.25% w/w to protect dry un-infested, winnowed maize and sorghum grain that is to be stored on-farm in sacks or woven granary baskets for periods of 4 months or more in Tanzania.

The test weight (bulk density) of untreated sorghum used in experiments was 76.74 kg/hl. After mixing with 1,000 ppm of Protect-It, test weight was reduced by 1.5 kg/hl, or by 1.95% (Table 3), significantly less if compared with the effect of DE on the reduction of test weight of other cereals [14].

Jackson and Webley [15] investigated the effect of diatomaceous earth on physical properties relating to the handling and storage behavior of 14 different commodities, including sorghum. Seven application rates of DE were applied to the various commodities and the effects on test weights (bulk density) and flow rates (flowability) were measured. The magnitude of test weight and flow rate effects varied by commodity and was greatest for maize followed by wheat. The effects of DE on sorghum rated among the oilseeds, which were the least influenced by DE. Using 500 ppm, the test weight for maize was reduced by 8.9%, for wheat 7.7% and for sorghum 3.7%. The flow rate for maize changed 39% while for sorghum it changed by 11%.

5. Conclusion

Under laboratory conditions the concentration of 1,000 ppm (2 pounds/ton) for treating sorghum should yield high mortality for all stored sorghum pests and prevent the damage caused by infestation. This concentration should control at least 90% of the most DE tolerant species, the lesser grain borer and the red flour beetle, and give 100% control for the rice weevil. Complete control of all species (100% mortality) in 2-3 weeks on sorghum (13% m.c. or less) can be achieved by applying 1,500 ppm (3 pounds/ton) of Protect-It.

DE Protect-It can protect stored sorghum grains and prevent the damage caused with the most dangerous and worldwide distributed insect pests with the application of 1000 to 1,500 ppm while having a minimal effect on sorghum bulk density (test weight).

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Different Carbon Fractions in Soils and Their Relationship with Trace Elements Content

Lubica Pospisilova, Petr Skarpa and Marie Konecna

Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of Agriculture, Mendel University in Brno, Zemědělská 1, Brno 613 00, Czech Republic

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Abstract: Luvisols, Stagnogleys and Cambisols, although less fertile, are used intensively for mixed farming, grazing and as forestland. Therefore we aimed our study at determination of total organic carbon (TOC) content, humic substances (HS) content, humic acids (HA) content, fulvic acids (FA), hot water extractable carbon (Chw) content and content total and labile trace elements content. Humic substances quality was assessed by HA/FA ratio and by coloured indexes measured in ultraviolet and visible UV-VIS spectral range. The total and labile contents of Zn, Cd, Cu, Co, Pb, Mo and Se were determined by flame or electro-thermal atomic absorption spectrometry after extraction of the soil samples in the aqua regia (total content) and in the solution of 0.01 M $CaCl_2$. Total and labile trace elements content was correlated with determined carbon fractions and soil reaction. Results showed that studied soils content low amount of TOC and had low quality of humic substances. HA/FA ratio was less than 1 and colour indexes were higher than 4. All determined carbon fractions correlated with labile form of Zn and Cd. Correlation between soil reaction and total zinc content was found. Significant effect of humic substances content on to water-soluble forms of heavy metals was detected.

Key words: Humic acids, fulvic acids, hot water extractable carbon, humus fractionation, labile trace elements, total trace elements.

1. Introduction

Soil organic carbon undergoes short and long term transformation. Under a dynamic equilibrium a portion of organic carbon is mineralised, and the same portion is newly formed. This portion is considered as a labile organic carbon (Chw). As stabile carbon fractions we considered total organic carbon (TOC), humic substances carbon (HS), humic acids (HA) and fulvic acids carbon (FA). The HA fraction consist of hydroxyphenols, hydroxybenzoic acids, and others aromatic structures with linked peptides, amino compounds, and fatty acids. Fulvic acids are typically composed of a variety of phenolic and benzene carboxylic acids. FA molecule held together by hydrogen bonds to form stable polymeric structures or by association with polysaccharides that are easily separated by adsorption on charcoal or by gel chromatography. FA contains more oxygen, less carbon and more acidic functional groups, particularly -COOH comparing with HA. Humins are considered to be insoluble fraction consisting of the humic type polymers that form strong associations with minerals as quoted in Refs. [1-3].

Labile carbon fraction is considered as an important factor for evaluation of anthropogenic influence. Many authors reported that the earliest detectable effects of changes in the supply of organic matter to soils are most readily mineralisable fraction [4-7]. Soil microorganisms through their enzymes are able easily to mineralise labile carbon sources while the rate of this process is different. Labile and stabile carbon fractions also play an important role in processes of pollutants transport and bioavailability. Associations between dissolved organic carbon, heavy metals and others hydrophobic pollutants were studied by M.H.B. Hayes,

Corresponding author: Lubica Pospisilova, Ph.D., research field: soil science. E-mail: lposp@mendelu.cz.

N. Senesi, R. Wandruszka et al. [3, 8, 9]. They showed significant connection between carbon fractions, content, soil texture, soil reaction and trace elements content. Decreasing of total organic carbon and soil acidification caused serious problems with soil chemical properties and the consequent loss of soil quality. Elevated CO_2 emissions from cultivated soils have lately encouraged proposals to control organic carbon stability by carbon sequestration in terrestrial ecosystems. Possible increase of sequestrated carbon today is low and represents about 0.7 % of net primary production [10].

The main contribute of our work was to give evidence that humic substances play an important role not only in soil fertility but also fulfil an important environmental function and are accepted to participate in binding of trace elements. We try to find correlation between trace elements content and different carbon fractions in soils with different type of land use.

2. Materials and Methods

During the period 2008-2010, we determined content of different carbon fractions in selected subtypes of Luvisols, Stagnogleys and Cambisols. All selected localities are in the foothills of the Hrubý Jeseník Mts. and occur in places higher than 300 m. a. s. l. Characterisation of selected localities and soil types are given in Tables 1 and 2. Objects of our study were arable soils and grassland: (1) *Gleyic Luvisol* (Šumperk, variant 1 - arable soil); (2) *Gleyic Luvisol* (Šumperk, variant 2 - arable soil); (3) *Gleyic Luvisol* (Vikýřovice - arable soil); (4) *Haplic Luvisol* (Bratrušov - arable soil); (5) *Haplic Stagnogley* (Bílčice - grassland); (6) *Haplic Cambisol* (Rapotin - grassland); (7) *Haplic Stagnogley* (Sluneční - grassland); (8) *Haplic Cambisol* (Přemyslov - grassland). Soils were sampling in the upper layer (0-0.20m, or 0.05-0.20m) twice a year. Basic soil parameters were determined by methods given bellow. Soil reaction was determined by the potentiometric method in distilled water and in KCl solution. Particle size analysis of soils was determined by the pipette method. Total organic carbon content (TOC) was determined by wet digestion according to Ref. [11]. Fractional composition of HS was determined according to Ref. [12] as follows: 5 g of air dried soil sample, sieved at mesh size of 1 mm and extracted by a mixture (1:1; 0.1 M NaOH + 0.1 M $Na_4P_2O_7$) for 24 h. The sediment was separated by centrifugation at 2,800 g for 10 min, washed with mixture and centrifuge again. Two individual washings were unified with original supernatant, acidified with concentrated H_2SO_4 to pH = 1.5. We allowed to precipitate HA overnight. Sum of HS, HA and FA was determined by titrimetric method in aliquot volumes. Ultraviolet-visible spectroscopy of prepared soil samples extracts, after filtration were performed by Varian Cary 50 Probe spectrometer with optical fibre within the range 300-700 nm. Colour indexes ($Q_{4/6}$) and humification degrees (HD) were calculated according to Ref. [13]. Labile carbon (Chw) content was determined by hot water extraction method according to Körschens [4, 5]. The total and labile contents of selected trace elements were determined by flame or electro-thermal atomic absorption spectrometry after extraction of the soil samples in the aqua regia (total content) and in the solution of 0.01 M $CaCl_2$ (labile forms) using Analytic Jena ContrAA 700.

3. Results and Discussion

Studied soils varied in total organic carbon (TOC) and labile organic carbon (Chw) content depending on management systems (Fig. 1). Both determined carbon forms (TOC and Chw) were in higher amount in grassland (Figs. 1 and 2), except *H. Stagnogley* (locality Sluneční). Lower TOC values in arable soils could be explained by higher mineralization rate. Soil reaction was acid, except *Gleyic Luvisol* in Vikýřovice and *Haplic Luvisol* in Bratrušov, where soil reaction was neutral. Soils were silt-loam and sand-loams textured, with medium CEC and low TOC content. Humic substances quality was low and HA/FA ratio was less than 1, except *Gleyic Luvisol* in Vikýřovice. In

fractional composition of humus it is evident the prevalence of fulvic acids (Table 3). Absorbance of HS isolated from soils under different management systems is given in Figs. 3 and 4. Results showed higher absorbance and HS quality in arable soils to compare with grassland. Calculated colour indexes suggested the same situation (Table 3). Higher values of $Q_{4/6}$ corresponded with lower HS quality. Our findings corresponded with literature data given by M. Körschens, L.B. Guo, A. Albrecht et al. [4, 5, 14, 15]. Distinctive variability of labile trace elements content due to soil type and land use was found (Table 4). Higher labile trace elements content was determined in grassland soils to compare with arable soils. Correlation

Table 1 Selected localities and their characterization.

Locality	Soil types	Management	GPS N	GPS EO	m.a.s.l. (m)
Šumperk (Š, 1)	G. luvisol	arable	49°97´42´´	16°96´88´´	329
Šumperk (Š, 2)	G. luvisol	arable	49°97´29´´	16°96´80´´	329
Vikýřovice (V)	G. luvisol	arable	49°96´80´´	17°00´55´´	319
Bratrušov (BR)	H. luvisol	arable	49°07´44´´	16°59´28´´	340
Bílčice (BI)	H. stagnogley	grassland	49°86´79´´	17°54´59´´	605
Rapotín (R)	H. cambisol	grassland	50°00´32´´	17°00´83´´	390
Sluneční (S)	H. stagnogley	grassland	50°11´05´´	17°05´73´´	700
Přemyslov (P)	H. cambisol	grassland	50°10´54´´	17°05´15´´	800

Table 2 Soil reaction and texture classes in studied soils.

Soil types	pH/H_2O	pH/KCl	Texture classes (%)		
			Sand 2.00-0.05 mm	Silt 0.05-0.002 mm	clay < 0.002 mm
G. luvisol (Š, 1)	7.0	5.8	19.44	62.6	17.96
G. luvisol (Š, 2)	7.0	5.9	17.88	64.66	17.46
G. luvisol (V)	7.1	6.8	34.66	48.3	17.04
H. luvisol (BR)	6.8	6.2	24.74	58.40	16.86
H. stagnogley (BI)	5.0	4.4	29.16	57.28	13.56
H. cambisol (R)	6.5	5.7	57.86	32.76	9.38
H. stagnogley (S)	5.7	5.3	48.8	40.44	10.68
H. cambisol (P)	6.0	5.2	58.3	34.02	7.68

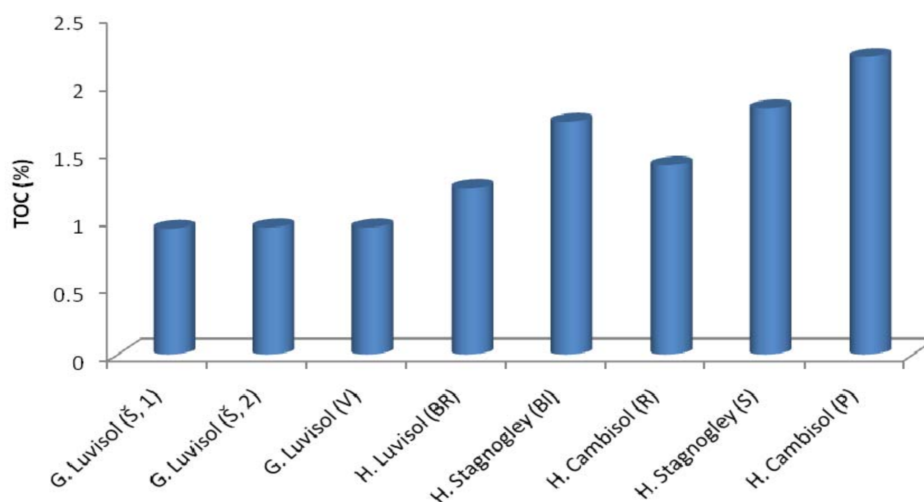

Fig. 1 Average total organic carbon content in studied soils.

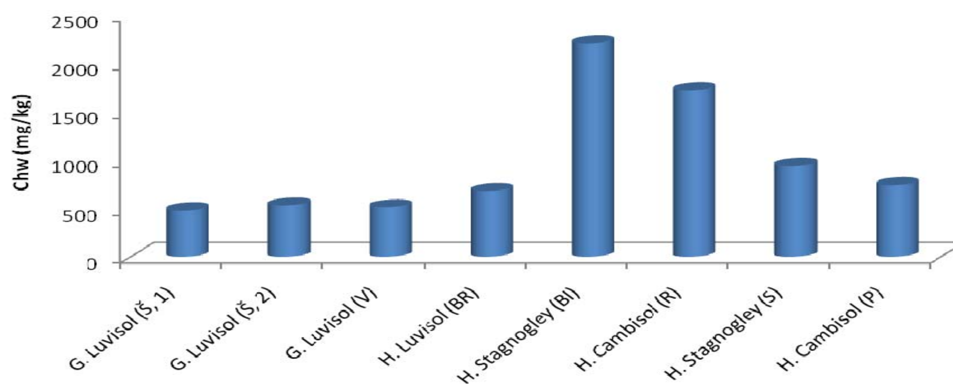

Fig. 2 Average labile carbon content in studied soils.

Table 3 Different carbon fractions content and humic substances quality in studied soils.

Locality	TOC (%)	Chw (mg/kg)	HS (mg/kg)	HA (mg/kg)	FA (mg/kg)	HA/FA	$Q_{4/6}$
G. Luvisol (Š, 1)	0.93	480	5	2	3	0.7	5.8
G. Luvisol (Š, 2)	0.94	530	5	2	3	0.7	5
G. Luvisol (V)	0.94	510	6	3	3	1.0	5.4
H. Luvisol (BR)	1.23	682	5	2	3	0.7	7.8
H. Stagnogley (BI)	1.72	2,212	9	4	5	0.8	9
H. Cambisol (R)	1.4	1,725	5.5	2.5	3.5	0.7	6
H. Stagnogley (S)	1.8	940	7	3	4	0.75	8
H. Cambisol (P)	2.2	742	9	4	5	0.8	7

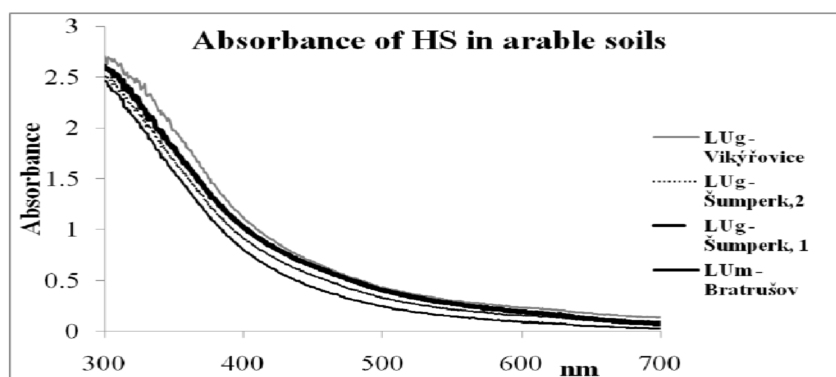

Fig. 3 UV-VIS spectra of HS isolated from arable soils.

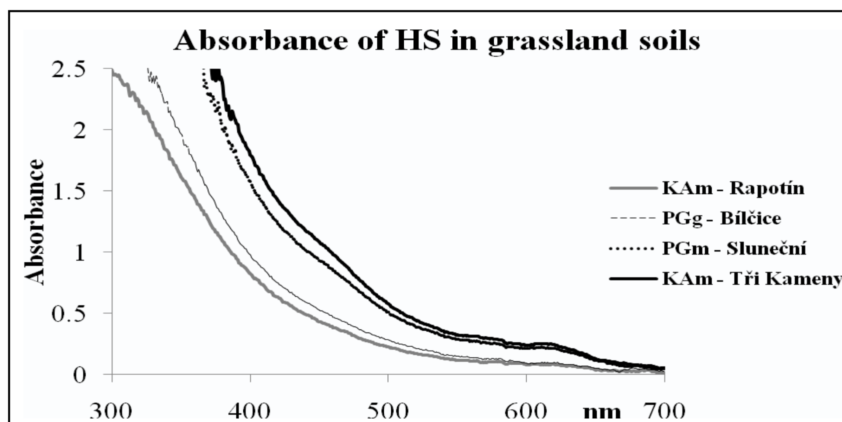

Fig. 4 UV-VIS spectra of HS isolated from grassland.

Table 4 Labile trace elements content determined in 0.01 M $CaCl_2$.

Soil types	Labile trace elements forms (0.01 M - $CaCl_2$) (mg/kg)						
	Zn	Co	Cu	Pb	Cd	Mo	Se
G. luvisol (Š, 1)	0.101	< 0.003	0.058	< 0.012	0.008	< 0.007	< 0.04
G. luvisol (Š, 2)	0.040	< 0.003	0.050	< 0.012	< 0.003	0.008	< 0.04
G. luvisol (V)	0.055	0.005	0.063	< 0.012	0.006	< 0.007	< 0.04
H. luvisol (BR)	0.028	< 0.003	0.095	< 0.012	< 0.003	< 0.007	< 0.04
H. stagnogley (BI)	0.452	0.074	0.075	< 0.012	0.0475	< 0.007	< 0.04
H. cambisol (R)	0.061	0.033	0.034	< 0.012	0.0206	< 0.007	< 0.04
H. stagnogley (S)	0.535	0.051	0.028	< 0.012	0.0487	< 0.007	< 0.04
H. cambisol (P)	0.176	0.006	0.024	< 0.012	0.0089	< 0.007	< 0.04

Table 5 Correlation between labile trace elements content and different carbon forms (r = 0.632; α = 0.05; n = 8).

	Co	Cu	Pb	Cd	Mo	Se	Zn
HS sum	0.494	-0.5722	0	0.6599	-0.3374	0	0.7632
HA sum	0.1518	-0.5001	2.61E-16	0.3305	0	0	0.4808
TOC	0.5632	-0.44471	3.673E-17	0.6683	-0.3404	0	0.7995
Chw	0.2729	3.7E-17	0	0.4837	0.2208	0	0.686

between different carbon forms (TOC, Chw, HS sum and HA sum) and labile Zn and Cd was found (Table 5). Further we follow relationship between soil reaction and trace elements content. Our results corresponded with data given by Mosquera-Losada et al. [16]. They reported a significant effect of soil reaction and fertiliser application on labile Zn and Co content. Our results showed high correlation coefficient -0.6253 and the strong effect of pH on total zinc content in soil (Fig. 5). Similar results were also published by Chukwuma et al. [17]. Both showed that after mineral fertiliser application total zinc content significantly ($P < 0.01$) correlated with pH and the same trend was monitored in the treatment with slurry [16, 17]. However, according to our results a significant dependence ($P < 0.05$) on the soil reaction was discovered only with zinc. We also came to the conclusion that the sum of humic substances, sum of humic acids and fulvic acids had a significant effect ($P < 0.05$, $P < 0.01$) on the content of water-soluble forms of heavy metals.

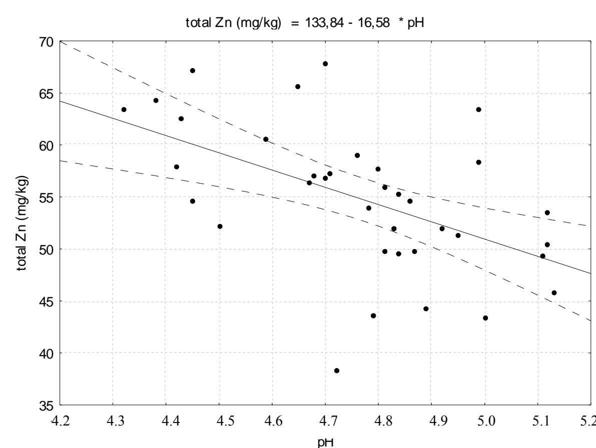

Fig. 5 Correlation between soil reaction and total zinc content.

Soil pedogenetic characteristics, type of land use and soil reaction were the main factors influencing content and quality of carbon forms and bioavailability of studied trace elements. For better understanding of the effect of land use change on soil carbon sequestration is highly recommended use of broad range of long-terms field experiments results.

4. Conclusion

We can conclude that humic substances content and quality strongly influenced the total and labile trace elements content in soils. Soil genesis, land use and soil reaction were the next very important factors influencing trace elements content and bioavailability. Determined carbon fractions significantly correlated with water-soluble

forms of zinc and cadmium.

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Magnetic Stimulation Replacing Fertilizers Causing Health Hazards in Plants

Ran Vijoy Kumar Charan

Department of Physics, Gaya College, Gaya-under Magadh University, Bodhgaya Bihar 823001, India

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Abstract: Cow-dung or compost which was earlier used is now replaced by chemical fertilizers to improve the fertility of land. But such fertilizers clearly pollute the plant and the yield. To eliminate such health hazards, an experiment was done by placing a plant inside an electromagnetic solenoid which has an arrangement to apply oscillatory magnetic field. It has been found that leafs are wilted due to loss of water from roots (since plucked from ground) exhibiting xanthophyl synthesis after about 60 minutes of oscillation. This phenomena clearly shows an increase in rate of ascent of the sap and hence the pulse rate. This action follows due to the resultant dominating effect of its diamagnetic water (90-95%). Now, if the field is made stimulatory, the pulse rate becomes abrupt, which in turn increases the growth of the plant and its yields. Therefore, if a farming-field is divided into several small sections with arrangements of applying stimulatory magnetic field, then the plant's growth becomes almost similar as found by use of fertilizers. Hence, the stimulatory magnetic field can take over the use of fertilizers in plant's growth, saving us not only from their health hazards but also from millions of rupees spent in their production.

Key words: Cow dung, hazards, stimulated, solenoid, retentivity, hysteresis, oeresteds, transpiration.

1. Introduction

In earlier days, cow-dung or compost were used as natural fertilizer to improve the fertility of the land, which didn't pollute the plant or its yields. But nowadays, due to decreasing fertility of the land owing to the global warming, excess carbon emission, and green house gas problem, decreasing magnetic moment of earth for its increasing distance from its galaxy and so many other reasons, the growth of plants and their yields are badly affected. So we are using chemical fertilizers formed of so many organic and inorganic materials to improve the fertility which not only pollute the plants but also poison their yields i.e. fruits, vegetables, etc. Thus, a great health hazard is created for humans, which causes so many serious diseases.

To eliminate such health hazards, a process is developed here through an experiment by applying stimulated magnetic field (in addition to the natural terrestrial magnetic field) to a plant whose pulse rate and hence the growth is found to be increased without use of any fertilizers.

Corresponding author: Ran Vijoy Kumar Charan, Ph.D., research field: plant science. E-mail: rvkcharan@rediffmail.com.

2. Experiment View and Result

An experiment was done with a sample of mesophytic plant by keeping it in an electromagnetic solenoid, in which there was an arrangement to apply oscillatory/stimulatory magnetic field. A special circuit was used for the purpose as shown in Fig. 1.

The circuit arrangement was composed of IC 555 powering the electromagnetic solenoid (for placing the plant) through an electromagnetic relay to generate oscillatory magnetic field inside the solenoid. However, the field could be made stimulatory by setting the relay timing. The intensity [1] of such applied oscillatory magnetic field in the solenoid is given by the Eq. (1) below:

$$H_{OSC} = 4\pi n\left(I_{rise} - I_{discharge}\right) \quad (1)$$

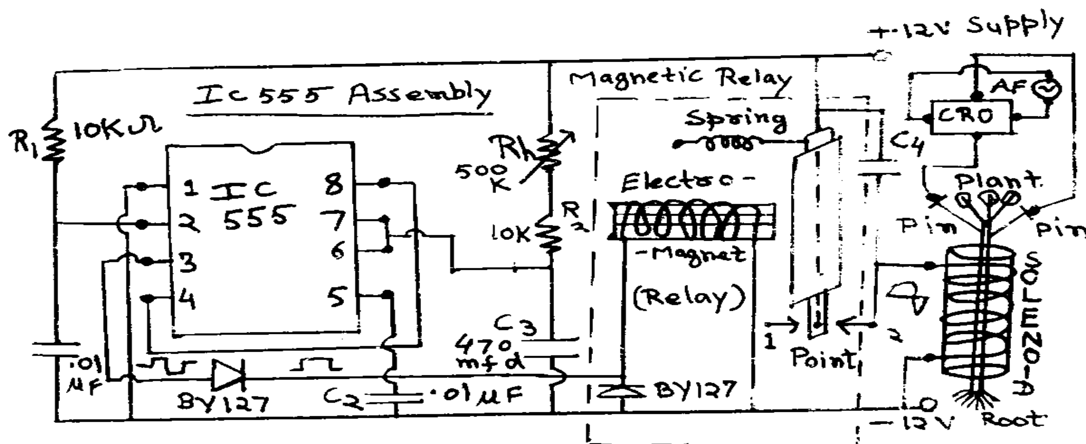

Fig. 1 Circuit for applying oscillatory magnetic field on plant in solenoid.

Now, by keeping the solenoid d.c. resistance R << L (inductance) and neglecting the higher term of $R^2/4L^2$ the field can be expressed in Eq. (2) below:

$$H_{OSC} = 4\pi n\left[\left\{I_0\left(I - e^{-t/\lambda}\right)\right\} - \left\{2\pi f Q_0 e^{-t/2\lambda} Sin 2\pi f t\right\}\right] \quad (2)$$

where n = no. of turns per unit length, I_o = maximum given current, Q_0 = steady charge at discharge, and f, λ & t are oscillation's frequency, wavelength & time respectively. Now a mesophytic plant for experiment was plant kept inside the solenoid another similar comparing plant was kept outside. The circuit was then made active to apply the square pulsating oscillatory magnetic field to the experimental plant. It was found that, due to effect of the oscillatory magnetic field [2], the rate of ascent of the sap was increased with the increase of the pulse rate. Further, at an optimum value of the frequency at which the oscillations become sharply damped and maximum the field was found to be almost stimulatory and the pulse rate was found to be increased abruptly. The optimum frequency was found to be different for different plants, obviously, because of their changed composition. It was further observed that, due to the increased rate of pulsation, the loss of water owing to rapid transpiration became excessive. So as the water content in the soil attached to the experimental plant was limited, it became slowly exhausted after 45 minutes of the oscillation, and the leafs were started wilting marking the beginning of the xanthophylls synthesis. After further 15 minutes of the oscillation, i.e. total of 60 minutes of the oscillation, all leafs were finally wilted. The experiment was then repeated with the plant in the farming field, so that the regular supply of water to the root was maintained. It was found that, at the optimum frequency of the oscillation after the aforesaid time, the growth of the plant was increasingly excited. The excitement in the growth can be maintained by applying the field for regulated time at preset intensity. In the experiment, the frequency was kept at 40-60 c/s, the magnetic retentivity intensity was kept at 5.19 oeresteds and the total magnitude of the field was kept at 180 oeresteds. The pulse rate was measured by injecting two fine probes inside the cortical zone (made of parenchyma) of the experimental plant kept inside the solenoid through analysis of Lissajous figures on oscilloscope.

3. Analysis of Result

We know that a plant's metabolism [3, 4] consists of 90-95% of water which was diamagnetic and the rest 5-10% contained several metals and non-metals para and ferro-magnetics but in minute form. Hence, in an oscillatory magnetic field, the magnetic moments of the spinning electrons of dia-magnetic materials revolving round the nucleus in one direction was balanced by that of the electrons spining in other direction, making the resultant magnetic moment to be zero which set their oscillations in perpendicular direction to the field, i.e. opposing the field according to dehass Van Alphan theory [5]. But in para &

ferro-magnetic materials, the magnetic moments of spining electrons were not balanced accordingly due to availability of some unpaired electrons. This phenomena created residue magnetization which set their oscillations in direction of the field as the Curie and Wiess law [6].

Thus, it was found that only the diamagnetics oscillated freely and hence dominated in the result due to higher percentage in their metabolism where as the little amount para & ferro-magnetics oscillated with some magnetic retentivity i.e. not freely due to their little percentage, and so did not affect the result too much. This phenomena was well exhibited in Hysteresis-cum-oscillation curve in Fig. 2.

Thus, the plant got a resultant magnetic field. And these actions resulted in magnetic stimulation of the plant which in turn increased the rate of ascent of sap and the pulse rate, and finally increased the plant's growth. But if the applied field was made sharply stimulated, i.e. damped oscillatory [7] at its optimum value, as shown in Fig. 3, the pulse rate and hence the growth was found to be abrupt.

The optimum value was found to be different for different plants due to their changed metabolism. However, a good relation can be established in between the pulse rate and the applied field.

4. Application of Result

The effect of the growth of plant by application of the stimulated magnetic field can be well harnessed in cultivation of crops, where the farming-field is divided into several small sections and each section has an

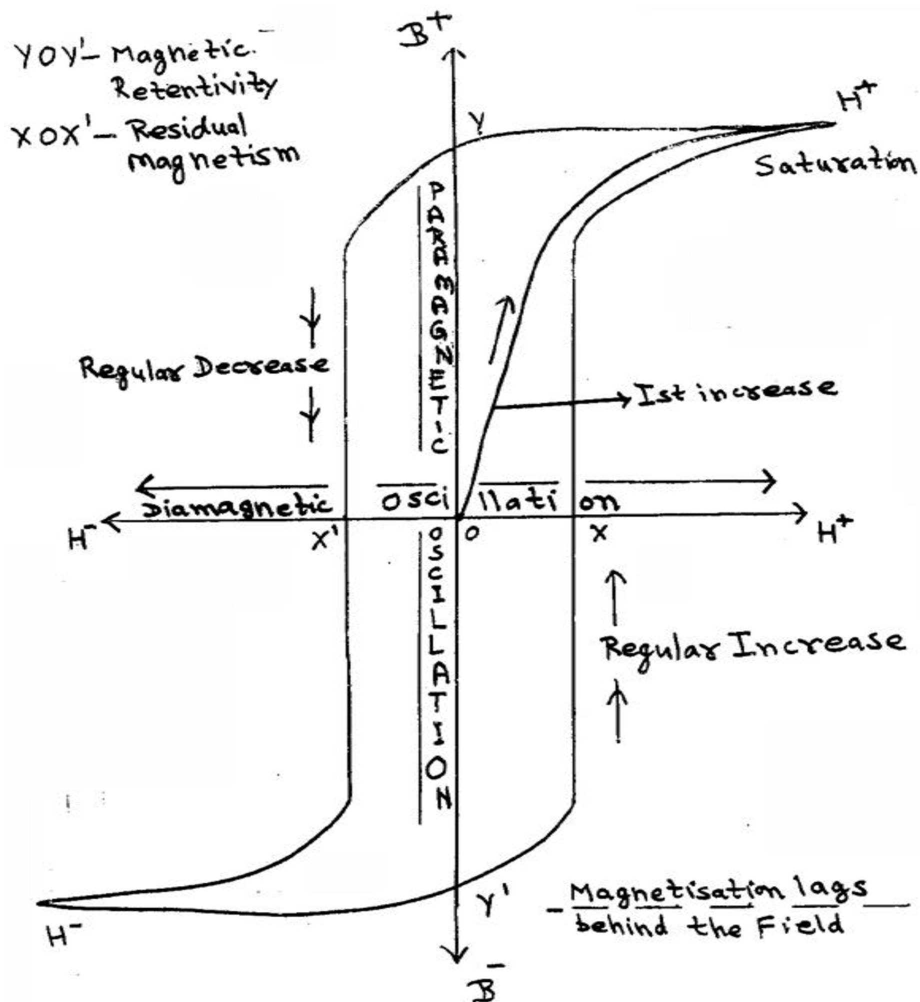

Fig. 2 Hysteresis curve for showing Dia and Para magnetics inter-perpendicular oscillations.

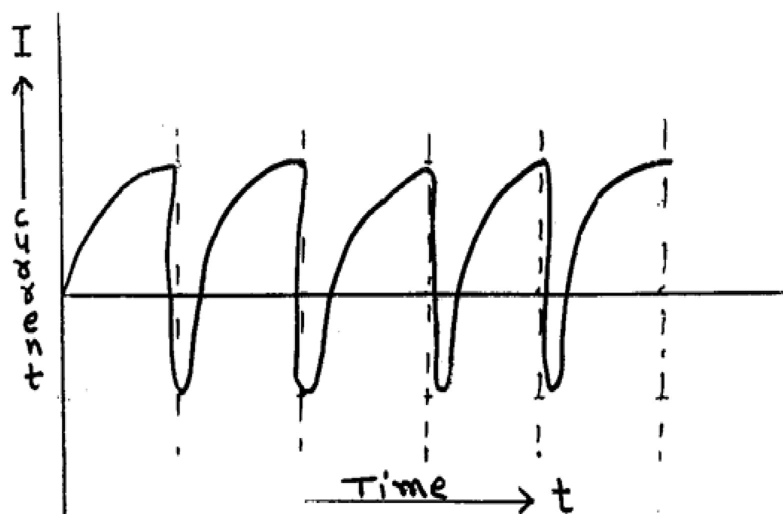

Fig. 3 Nature of damped oscillatory current inside plant kept in solenoid.

arrangement of applying the field along with its normal nourishing, i.e. watering, etc. It is found that, after application of the field for certain pre-set time, the growth of the plants become almost similar and satisfactory as found by use of so many fertilizers. The growth can be more excessive if the optimum frequency of each variety of plant is known through the earlier experiments and then applied to the plant.

5. Conclusion

Thus it is inferred that by replacing the fertilizers through the application of the stimulating magnetic field, we are not only saved from their health hazards causing so many diseases, but also million of rupees spent in their production. However, a larger and more precise arrangement is needed for better result.

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