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The Effects of Trehalose on the Bioluminescence and Pigmentation of the Phase I Variant of *Photorhabdus luminescens*

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Abstract: *Photorhabdus luminescens* is a Gram-negative, bioluminescent, pigment producing enteric bacterium, which is pathogenic to insects and has the capability to undergo phase variation. The phase I variant of *P. luminescens* exists as a mutualistic symbiont where it plays a critical role in the life-cycle of the soil-dwelling nematode, *Heterorhabditis bacteriophora*. Both the bacterium and the nematode receive their nutritional requirements from the bioconversion of the insect host which is rich in many macromolecules such as the disaccharide, trehalose. Trehalose is a non-reducing disaccharide of glucose that is formed by an α-1,1-glycosidic bond and is associated with the physiology of many bacteria, insects and nematodes. Trehalose has been shown to be the most abundant storage sugar found within insect hemolymph (1%-2%). The physicochemical properties of trehalose allow this carbohydrate to act as a stress protectant where it has been implicated with thermal stress, dehydration, and osmotic protection of many microorganisms. Due to these properties, trehalose may allow culture stability of the phase I variant *in vitro* and *in vivo*. Traits of the phase I variant that were studied in this work include bioluminescence and the production of the red anthroquinone-derived pigment. The carbohydrates that were utilized in this study were glucose and trehalose; where shake flask cultures of the phase I variant were cultured at room temperature for up to six days in carbohydrate supplemented basal media with increasing carbohydrate concentrations of 0.1%, 0.5% and 1.0% (v/v). Relative luminosity, pigmentation and pH were graphed as a function of time, carbohydrate used, and carbohydrate concentration. Data obtained from this study suggests that the supplementation of 1.0% trehalose, when culturing the phase I variant of *P. luminescens*, can maintain bioluminosity and pigmentation over extended periods of time (five days) as compared to basal media and basal media supplemented with 1.0% glucose.

Key words: Trehalose, *Photorhabdus luminescens*, pigmentation, bioluminescence, *Heterorhabditis bacteriophora*.

1. Introduction

Trehalose is a naturally occurring non-reducing disaccharide that is formed by α-1,1-glycosidic linkage of two glucose molecules (Fig. 1). This linkage makes trehalose very resistant to acid hydrolysis, stable at extreme temperatures, and resists cleavage by many α-glycosidases [1]. Because of these properties, trehalose, like glycerol, is being utilized to stabilize lipids and proteins and can also be used to stabilize more complex biologicals such as viruses, bacteria and tissues [2, 3]. Trehalose has also found numerous applications in commercial industries, in particular

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Trehalose can be synthesized and utilized by many organisms, including bacteria, fungi, plants, and many invertebrate animals such as nematodes and insects [6-9]. In a 1957 report, researchers were able to isolate crystalline trehalose from nematodes of the genus *Ascaris* and had also demonstrated the presence of trehalose in other human parasitic worms [10]. In insects, trehalose has been found as the most abundant sugar (1.0-2.0% w/v) within the insect hemolymph where it is used as a quick energy source for flight [11]. Trehalose concentration in the hemolymph of different insects generally varies from 10 to 50 mM; however, lower concentrations have been reported and the highest concentrations occur in lepidopteran and coleopteran insects where the concentrations exceed 100 mM [12]. In lepidopterous insects, trehalose accounts for more than 90% of the total blood sugar. For example, the Greater Wax moth, *Galleria mellonella*, a lepidopteran insect, has a blood trehalose concentration of about 1.5% (w/v) and a glucose concentration of 0.02% (w/v) [13].

*Photorhabdus luminescens* is a Gram-negative, enteric bacterium that is pathogenic to most insects and lives in a symbiotic relationship with the soil-dwelling nematode, *Heterorhabditis bacteriophora* [14]. This bacterium has the ability to phase shift, meaning that it is able to shift between two metabolically different states, phase I and phase II [15]. It is known that the phase I variant of this bacterium is able to support the life-cycle of the nematode; however, the phase II variant cannot support nematode growth *in vivo* or *in vitro* [16]. Under laboratory conditions, it is difficult to maintain the phase I variant due to the bacterium’s natural tendency to shift into phase II [17].

In order to manage the rate of phase variation in *P. luminescens*, reasons and mechanisms of this microbiological phenomenon must be completely understood. In most pathogenic bacteria phase variation is an adaptation that ensures bacterial survival in response to extreme environmental stresses such as temperature, osmolarity, oxygen concentration, and pH [18, 19]. In comparison with *E. coli*, *P. luminescens* does indeed respond to osmotic conditions; however, the biological responses between the two species of bacteria are different. In unfavorable osmotic conditions, *E. coli* responds by the over-production of cytoplasmic trehalose [20], whereas the phase I variant of *P. luminescens* shifts its metabolic activities to a more stable state, phase II [21].

This study examines the effects of glucose and trehalose at various concentrations on the stability of bioluminescence and pigmentation of the phase I variant in liquid culture. These phenotypic traits were utilized as they are primarily expressed by the phase I variant whereas expression diminishes upon transitioning to the phase II state.

**2. Materials and Methods**

**2.1 Materials and Supplies**

2.1.1 Preparation of Reagents and Culture Media

Nutrient broth (3 g of beef extract and 5 g of digested gelatin per liter of water), distributed by Carolina Biological Supply Company (Burlington, North Carolina, USA), was used as the basal media and pH was adjusted to 7.00 ± 0.01. Stock solutions of glucose and trehalose were prepared at 20% concentrations and filter sterilized using a 0.2 micron syringe filter. Experimental media containing either glucose or trehalose at 0.1%, 0.5%, and 1.0% (v/v) concentrations were prepared by adding the required amount of the respective stock solutions to the basal medium. Five milliliters of liquid basal media was aliquoted into four 15 mL screw-cap culture tubes, sterilized and used for overnight cultures of the phase I variant.

Experimental media containing either glucose or trehalose at 0.1%, 0.5%, and 1.0% (v/v) concentrations were prepared by adding the required amount of the respective stock solutions to the basal medium. Five milliliters of liquid basal media was aliquoted into four 15 mL screw-cap culture tubes, sterilized and used for overnight cultures of the phase I variant.

Nutrient, NBTA and MacConkey agar plates were prepared per liter (w/v) of distilled water as follows for the determination of phase variants of *Photorhabdus luminescens*:

- Nutrient agar (NA)—3 g of beef extract, 5 g of digested gelatin, 15 g of agar;
- NBTA [22]—nutrient agar supplemented with
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0.0025% (w/v) bromothymol blue and 0.004% (w/v) 2,3,5-triphenyltetrazolium chloride;

- MacConkey (MAC)—17 g of digested gelatin, 1.5 g of digested casein, 1.5 g of digested animal tissue, 10 g of lactose, 1.5 g of bile salts, 5 g of sodium chloride, 0.03 g of neutral red, 0.001 g of crystal violet and 13.5 g of agar.

2.1.2 Creation of a Qualitative Scale for Determining Pigmentation

A qualitative pigmentation scale was created to observe numerical changes in culture pigmentation. Three types of media were used to create various shades of pigmentation (Fig. 2) after culturing the phase I variant for a two-day period at 25 °C are shown below:

- Nutrient broth No. 1—15 g peptone, 3 g yeast extract, 6 g sodium chloride, and 1 g D-glucose, distributed by Fluka Analytical, displayed a dark yellow pigmentation (score of 1);
- Nutrient broth—3 g of beef extract and 5 g of digested gelatin, distributed by Carolina Biological Supply Company exhibited an orange pigmentation (score of 6);
- Brain heart infusion broth—7.7 g calf brain infusion, 9.8 g beef heart infusion, 10 g proteose peptone, 5 g sodium chloride and 2.5 g disodium phosphate, distributed by Presque Isle Cultures, presented dark red pigmentation (score of 12).

Various shades of pigmentation between these above mentioned scores were obtained by manipulating concentrations (w/v) of each media to obtain other pigmentation scores.

![Fig. 2 Media used to create a qualitative scale for scoring culture pigmentation.](image)

**Fig. 2** Media used to create a qualitative scale for scoring culture pigmentation. Left: Nutrient broth No. 1 produced a pigmentation score of 1 (dark yellow); Middle: Nutrient broth from Carolina Biological exhibited a pigmentation score of 6 (orange); Right: Brain heart infusion without glucose displayed a score of 12 (dark red).

2.1.3 Isolation of *P. luminescens*

*Heterorhabditis bacteriophora* nematodes, labeled as NemaSeek, were obtained from ARBICO Organics, Tuscan, Arizona, USA. Larvae of the Greater Wax moth, *Galleria mellonella*, were obtained from Carolina Biological Supply Company, Burlington, North Carolina, USA. *P. luminescens* was isolated from *H. bacteriophora* infected larvae of *G. mellonella*. One gram of the nematode/packaging mixture was added to 10 mL of 0.25% hyamine in a 50 mL centrifuge tube. The nematode mixture was allowed to sit at room temperature for 5 min, centrifuged at 3,000 rpm for 3 min and slowly decanted the supernatant. An additional 10 mL of 0.25% hyamine was added and the above was repeated. Two volumes (20 mL) of sterile distilled water was added to the tube and inverted slowly five times to obtain homogeneity, centrifuged again for 3 min at 3,000 rpm and the supernatant was decanted. This rinse step continued twice more and the nematode pellet was resuspended in 5 mL of sterile distilled water and 0.5 mL of the suspension was added to the center of sterilized filter paper that had been inserted into a sterile, disposable Petri dish.

Ten larvae of *G. mellonella* were surface sanitized five times by brief pipetting with 0.25% hyamine. The larvae were rinsed 3 times with sterile distilled water by brief pipetting and placed onto sterile filter paper containing *Heterorhabditis* nematodes and incubated at ambient temperature for at least 2 days to observe for larval mortality. To determine the presence of the phase I variant of *P. luminescens*, phenotypic traits (luminescence and pigmentation) were used to screen for infected larvae. Larvae appearing red to brick-red in color were placed into a clear 1.5 mL microcentrifuge tube and luminosity was measured.

Infected larvae that exhibited pigmentation and high luminescence were used to obtain *P. luminescens*. Infected larvae were aseptically dissected, mid-sagittally, with a sharp, sterile blade and a
sterilized inoculating loop was used to obtain the infected insect hemolymph. The infected hemolymph was streaked onto NA plates for the isolation of phase I cells. Plates were then incubated at 25 °C for two days until red-pigmented colonies appeared. Labeled isolated colonies were streaked onto NBTA and MAC agars to verify the presence of phase I cells. According to Boemare and Akhurst, the adsorption of bromothymol blue in the presence of triphenyltetrazolium chloride within the NBTA media and the neutral red from the MAC plates, with the production of luminescence suggests the existence of phase I cells [15, 23].

2.2 Methodology

Four 5 mL overnight cultures of *P. luminescens* were prepared utilizing isolated colonies of the phase I variant and incubated at 25 °C for a minimum of 24 hours. After incubation, each overnight culture of the phase I variant was Gram stained to determine culture purity. A non-contaminated overnight culture was used to inoculate shake flasks containing control and experimental media at a 1.0% inoculum concentration. All shake flasks were incubated at room temperature and agitated on a platform shaker at 150 rpm. Just after thorough mixing (~ 2 min), samples of each flask were taken to establish a baseline (t = 0) for pigmentation, pH and luminosity. Samplings occurred in time sets that were scored and measured throughout a six-day period at random intervals.

Relative luminosity units (RLUs) were measured utilizing a Turner Biosystems Modulus™ single-tube luminometer using 1 mL samples of each culture. Culture samples were also subjected to pH determination utilizing a Fisher Scientific Accumet® AR10 pH meter standardized at room temperature (25 °C) with a slope of 95%. Culture pigmentation was scored visually on a numerical scale of one to 12 where the value of one is dark yellow and 12 dark red.

3. Results and Discussion

3.1 Results

3.1.1 Effects of Carbohydrates on Bioluminosity

Fig. 3A depicts supplemented basal media along with the control. For both glucose and trehalose cultures, maximum luminosity was reached (143,103 RLUs and 103,152 RLUs, respectively) within the first day. Luminosity of the 0.1% glucose culture increased at a rate of 159,674 RLUs·day⁻¹ and for the 0.1% trehalose culture the rate of increase for bioluminosity is 114,527 RLUs·day⁻¹, which is just slower than that of glucose by a factor of 0.7. As compared to the control, maximum luminosity of the basal media (56,423 RLU) was reached approximately 1.28 days with a rate of increase of 37,477 RLUs·day⁻¹. Fig. 3A also shows that luminosity of the glucose culture is declining more rapidly than the culture medium containing trehalose. The decline rate for bioluminosity of the glucose culture, when compared to the trehalose culture, is higher by a factor of 1.03. After five days of reaching maximum luminosity the ending RLUs for the glucose and trehalose cultures were 657.7 RLUs and 6,545 RLUs, respectively.

Results from basal media containing 0.5% of each carbohydrate utilized are shown in Fig. 3B. The glucose and trehalose supplemented media provide maximum luminescence within the first 24 hours of culturing (122,865 RLUs and 260,252 RLUs, respectively). The luminosity increase for the glucose supplemented media was determined to be 95,951 RLUs·day⁻¹, while the increase for trehalose was 207,390 RLUs·day⁻¹. When the two carbohydrates are compared, the rate of bioluminosity in the trehalose medium is higher than that of glucose by a factor of 2.16. When comparing these two carbohydrates, again trehalose is observed to maintain a higher luminosity (49,294 RLUs) than glucose (451 RLUs) at the ending of the experiment (six days). Glucose at this concentration exhibited a decline that is 109.3 times greater than that of trehalose.
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Fig. 3  Effects of carbohydrates on bioluminosity. A: 0.1% carbohydrates—trehalose depicted by the open circle maintained luminosity of an extended period of five days when compared to glucose (diamond) and basal media (triangle); B: 0.5% concentrations—trehalose (circle) shows luminosity that is approximately 10-fold higher than basal media (open triangle) and glucose (diamond); C: 1.0% concentrations—as depicted, trehalose (circle) did maintain high levels of luminosity (~ 1,000-fold greater) when compared to glucose and approx. 100-fold greater than the basal media. Also, when all trehalose cultures are compared, the 1.0% trehalose culture exhibited luminescence that is 10-fold greater than the 0.1% and 0.5% trehalose cultures.
Fig. 3C demonstrates that at 1.0% concentrations, glucose containing media reached peak luminosity (127,686 RLUs) within 24 hours and the trehalose supplemented media reached its peak (194,751 RLUs) within 30.7 hours (1.28 days). The rate of increase for the glucose culture was determined to be 159,674 RLUs-day⁻¹ and for trehalose 155,010 RLUs-day⁻¹. When compared to the trehalose culture, the factor of increase in luminosity for the glucose culture was calculated to be 1.03. At the conclusion of the experiment, trehalose was able to maintain a high level of luminescence, approximately 1.0 × 10⁵, over the experimental period when compared to the 1.0% glucose culture. The rapid decline of bioluminescence of the glucose culture was 1.46 times greater than that of trehalose. The final luminosity of the glucose medium (178.3 RLUs) was 374.3 times lower than that of the trehalose medium (66,744 RLUs).

3.1.2 Effects of 1.0% Carbohydrates on Culture Pigmentation and pH

In Fig. 4, pigmentation scores and pH are shown as a function of the carbohydrates used at 1.0% concentration and culture age. At the beginning of the experiment, the natural color of all the inoculated media was initially recorded as a 7 (on a 1-12 point scale) to obtain a baseline score for comparative purposes. Pigmentation increased over time when the phase I variant was cultured in basal media supplemented with trehalose. This pattern was also observed when the variant was cultured in basal media alone (control); however, when the phase I variant was cultured in media containing 1.0% glucose, pigmentation decreased over time. At the end of the experiment, the culture containing trehalose had a visual pigmentation score of 11 (deep-red) and the 1.0% glucose culture was visually scored one (dark yellow) indicating that another factor (culture pH) may affect the color and/or shade of the produced red pigment. To observe changes in culture pH, pH measurements were recorded and graphed as a function of time and the 1.0% carbohydrate used. At the beginning of the experiment the pH of each shake flask was measured to be approximately 7.00 ± 0.01. As culture age increased, the pH of the control media increased to a final pH value of 8.12 ± 0.01; whereas the trehalose culture increased to a final pH of 8.51 ± 0.01; and the glucose culture decreased to a final pH value of 5.53 ± 0.01 as shown in Fig. 4.

When the phase I variant was cultured in basal media, the initial pH of the basal medium (6.98 ± 0.01) gradually increased over the experimental period to a final culture pH value of 8.12 ± 0.01 ($r^2 = 0.993$). As the culture pH increased the culture pigmentation score also increased (Fig. 4A). Initial pigmentation was scored as a 7 and increased over time to a final score of 9. When cultured in basal media supplemented with 1.0% glucose, initial media pH (6.99 ± 0.01) gradually decreased over time to a final culture pH of 5.53 ± 0.01 ($r^2 = 0.992$). When the culture pH decreased, a decrease in the pigmentation score was also observed (Fig. 4B). The initial pigmentation score was seven and decreased over the experimental period to a final value of one. Before the bacterium was cultured in 1.0% trehalose supplemented basal media the initial pH was measured to be 7.00 ± 0.01. After inoculation, culture pH gradually increased over the six days period to a final culture pH value of 8.51 ± 0.01 ($r^2 = 0.992$). As observed above with the control medium, culture pigmentation of the trehalose medium also increased gradually (Fig. 4C). Initial pigmentation of the trehalose medium was scored to be seven and did increase to a final score of 11 after six days.

Pigment sensitivity to pH was further studied by the addition of 1 M hydrochloric acid (HCl) or 1 M sodium hydroxide to each culture (data not shown). Addition of 10 mL of 1 M HCl to the control culture (pH 8.12, pigmentation score of nine) led to a drop in pH to 5.62 and the pigmentation changed from red (score of nine) to light yellow (score of two). Subsequent addition of 1 M NaOH raised the pH to 7.96 and the pigment returned to light red (score of eight). Similar observations were seen with the trehalose culture. With
The Effects of Trehalose on the Bioluminescence and Pigmentation of the Phase I Variant of *Photorhabdus luminescens*

![Graph A](image1.png)  
**Fig. 4** Effects of culture pH on culture pigmentation over time.  
A: Basal media control depicting a gradual increase in culture pH as it seems to positively influence the increase of culture pigmentation; B: Basal media supplemented with 1.0% glucose depicts that the culture pH gradually decreased, negatively effecting culture pigmentation; C: Basal media supplemented with 1.0% trehalose is shown to have a gradual increase in culture pH which also positively effects the culture pigmentation to where the shade of pigmentation is deeper when compared to the control.

The glucose culture (final pH = 5.53, final pigmentation score = 1, dark yellow), 10mL of 1 M NaOH was added and the pH increased to 7.83 and a pigmentation score of eight (light red). After these observations 10 mL of HCl was added to the culture and the pH decreased to 5.48 and the pigmentation was scored as one (dark yellow).

### 3.2 Discussion

In insects, researchers suggest that trehalose is synthesized and stored within the fat body of insects and when needed is quickly released into the insect hemolymph at a rate of 252 µg of trehalose per mg of fat body per hour when needed for flight [24]. The enzyme trehalase hydrolyzes the α-1,1-glycosidic bond with stringent specificity, converting one molecule of trehalose into two molecules of glucose [25] that is ultimately used for high energy activities such as jumping and flying [12]. On the other hand, higher-level organisms produce another enzyme, trehalose phosphorylase that catalyzes the phosphorolytic cleavage of trehalose 6-phosphate into glucose 1-phosphate and glucose [26].

Trehalose 6-phosphate synthase is mainly responsible for the biosynthesis of trehalose 6-phosphate from UDP-glucose and glucose 6-phosphate whereby a subsequent dephosphorylation step by trehalose 6-phosphate phosphatase leads to the formation of free trehalose [27]. Reports have shown that trehalose biosynthesis serves an important role in *E. coli* for stress and osmoprotection [28, 29]. In *E. coli*, the trehalose biosynthesis operon is known to be induced by several factors including: (1) osmotic shock; (2) extreme temperatures; (3) desiccation; (4) entrance into stationary phase, and (5) the presence of maltose [20]. The trehalose biosynthesis operon from *E. coli* has previously been elucidated to genetically engineer plants to increase stress tolerance against dehydration [30, 31]. The synthesis of large amounts of trehalose within the cytoplasm has also been seen when *E. coli* was cultured in unfavorable osmotic conditions and that trehalose synthesis occurs independently of other carbon sources that are present in the medium, including trehalose itself [32].
The genome of *P. luminescens* contains a trehalose operon that encodes for a trehalose repressor protein (treR), the PTS system trehalose (maltose) specific transporter subunits IIBC (treB), and the enzyme trehalose 6-phosphate hydrolase (treC) [33]. This operon allows *P. luminescens* to utilize extracellular trehalose derivatives, such as trehalose 6-phosphate; however an operon responsible for trehalose biosynthesis in *P. luminescens* has yet to be identified, suggesting that this bacterium may not have the capability to produce cytoplasmic trehalose in response to unfavorable growth conditions [34]. To circumvent or adapt to environmental stresses, *P. luminescens* may utilize trehalose or trehalose-based derivatives found within the insect hemolymph for stress protection and/or a valuable source for carbon and energy [35].

3.2.1 Effects of Carbohydrates on Bioluminosity

Culturing the phase I variant of *P. luminescens* in basal media, and in basal media supplemented with 0.1% carbohydrates (glucose and trehalose) produced interesting results in regards to bioluminosity. When cultured in basal media alone (control), maximum luminosity was achieved at 30.7 hours when compared to glucose and trehalose supplemented media (~ 24 hours). With the glucose supplemented media a significant decrease in RLUs (658) was seen when compared to the control (1,041 RLUs); however, the culture containing trehalose showed a significant increase in RLUs (6,545) when compared to the control and glucose cultures. The significance of the trehalose containing culture is that the bioluminosity is 10-fold higher than the measured luminosity for the glucose media. This data indicates that luminosity may be stable in the presence of trehalose.

When compared to the control media, maximum luminosity for both 0.5% carbohydrate supplemented media was reached within the first day of culturing and decreased following the same linearity of the same media utilizing 0.1% carbohydrates; however the 0.5% glucose culture exhibited a slightly lower luminosity than reported with the 0.1% glucose medium. This decrease in luminosity of the 0.5% glucose medium may signify the increase of acidic end products from glucose metabolism [36]. Therefore a decrease in culture pH may affect the proteins required for bioluminescence. As for the trehalose supplemented media, luminosity is still shown to be approximately 10-fold greater than the luminosity of both the control and glucose media at the ending of the experimental period suggesting that another parameter may be involved.

Data obtained from both 1.0% glucose and 1.0% trehalose cultures suggest that utilizing trehalose to maintain bioluminosity does work really well. Through the use of 1.0% trehalose in the culture medium, trehalose did have a significant impact (66,744 RLUs) on the stability of luminosity which is almost 1,000-fold greater than the luminosity of the glucose media (178 RLUs). When comparing the glucose medium with the trehalose medium from a logical standpoint, the physicochemical properties of trehalose may be stabilizing the proteins required for bioluminescence [2-5].

The supplementation of the culture media with trehalose at increasing concentrations did outperform media supplemented with glucose. It can be seen that the use of trehalose enhances the culture luminosity as compared to basal media alone. At a 1.0% concentration, trehalose does maintain high culture luminosity over the entire experimental period (six days) and shows greater luminosity than the 0.1% and 0.5% trehalose cultures. The trehalose concentration of 1.0% is the minimum concentration that has been measured within the hemolymph of certain insects [11]. Future research is being planned to utilize trehalose concentrations up to 2.0%, the maximum reported concentration of trehalose found within insect hemolymph.

3.2.2 Effects of 1.0% Carbohydrates on Culture Pigmentation and pH

Culture pigmentation was another parameter that was used to compare the effects of 1.0% carbohydrates
over time. Initial pigmentation of all cultures was scored seven (color of the basal media) to which we were able to establish a baseline for comparative purposes. As time progressed, pigmentation of the glucose culture was decreasing and reached a final score of one while the trehalose culture exhibited an increase in pigmentation to a final value of 11 and the control reached a score of nine. From this data, we hypothesized that the changes in culture pH, resulting from carbohydrate metabolism, are responsible for pigment coloration, suggesting that this red anthroquinone-derived pigment is pH sensitive.

Data obtained from culture pH over the experimental period has brought a few things to light. Initial pH for all media used was within the neighborhood of 7.00 ± 0.01. Basal media and the 1.0% trehalose culture exhibited a similar pattern for pH. Over time for both cultures, pH gradually increased to final values of 8.12 and 8.51 (control and trehalose, respectively). On the other hand, the culture pH for the glucose medium gradually decreased to a value of 5.53. The pH of the basal media was expected to increase in pH since the media composition consisted of only beef extract and digested gelatin (sources of protein) and under this type of metabolism nitrogenous waste products are produced, which increase the culture pH [36].

As far as glucose is concerned, we expected to see a decrease in culture pH, in which we did, because through carbohydrate metabolism, acid end products are produced. The production of these acidic end products may explain the decrease in culture pH; however data for culture pH of the trehalose culture are puzzling. The breakdown of trehalose results in two glucose molecules and the utilization of these by bacteria should reduce the pH; however, the pH of the trehalose supplemented culture remained alkaline. This “phenomenon” may be attributed to slow substrate utilization/metabolism. To explore this in detail, the mechanism that involves trehalose metabolism must be further studied. The generated data, thus far, may suggest that trehalose metabolism occurs very slowly over time. This statement seems to be very true since this symbiotic bacteria and its nematode partner must stay within the insect cadaver for three-four weeks until the nematode population within the insect is stable or the insect is nutritionally depleted.

To observe the effects of pH on culture pigmentation as a function of time and carbohydrate used, graphs were created to visualize the correlation. When cultured in the control medium, the phase I variant had a natural tendency to slightly increase pH. When pH and pigmentation were compared, pigmentation increased with the increase in the pH value. A similar pattern was also seen when the bacterial cells were cultured in 1.0% trehalose medium. However, in the glucose medium the culture pH decreased along with the pigmentation score. Based upon analysis of the data obtained, there seems to be a correlation between pH and pigmentation. This correlation points out that the anthroquinone-derived pigment may be pH sensitive. After the crude experiment with the additions of HCl and/or NaOH to the cultures, color changes of the culture pigmentation were seen. In acidic conditions the red pigment becomes yellow and when the pH conditions are reversed the yellow pigmentation of the culture is converted back to its red coloration.

4. Conclusion

Trehalose, the “blood sugar” of insects, maintains stability of bioluminescence and pigmentation as a function of culture age, concentration and pH within a batch culture. It has been demonstrated that if the concentration of trehalose is increased, all of the phase I phenotypic traits studied are either enhanced, maintained, or both. Further experiments may indicate that a higher concentration of trehalose (1.5%-2.0%) may indeed maintain the stability, pigmentation and bioluminescence of the phase I variant of P. luminescens for extended periods of time that may be required for the nematode symbiont to develop and reproduce in vivo and in vitro.

Since trehalose is a disaccharide of glucose and upon
hydrolysis by trehalase, two glucose molecules are formed; however, it is puzzling to know why trehalose does not have the same effect as glucose alone. One suggestion that has been discussed is that the enzymatic rate at which trehalase converts trehalose into glucose is slower than the rate of protein catabolism. We suspect that protein catabolism by *P. luminescens* increases the culture pH. It is also possible that *P. luminescens* does not have the genetic potential to produce protein products that will allow the bacterium to utilize free trehalose. Furthermore, more studies involving the metabolism of trehalose and its derivatives must be performed to understand the role of trehalose in the microbial physiology of *P. luminescens*. Measurements of pH and pigmentation scores suggest that this red anthroquinone-derived pigment is sensitive to changes in pH. Due to its pH sensitivity, the pigment can be exploited to be used as a wide-range pH indicator. However, additional chemical analyses are needed to examine the chemical properties of this pigment.

### Acknowledgments

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### References


The Effects of Trehalose on the Bioluminescence and Pigmentation of the Phase I Variant of Photorhabdus luminescens


Intermediaries and Targets of the Oxidative Stress Induced by Natural Sunlight in *Escherichia coli*

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**Abstract:** The lethal effect produced by sunlight in bacteria depends on the photodynamic action of the ultraviolet component of the radiation. Neither the reactive oxygen species involved nor the targets for damage have been certainly identified, and the aim of this study was to address these issues. During exposures to natural sunlight, photo-protection provided by nitrogen sparging was compared with which provided by the addition of L-histidine (an efficient scavenger for hydroxyl radical and singlet oxygen) or mannitol (an hydroxyl radical scavenger which reacts poorly with singlet oxygen) to the irradiation medium. Both scavengers reproduced for the most part the effect of oxygen depletion, indicating that damage depends mainly on hydroxyl radical generation. Survival of irradiated bacteria decreased considerably when they were cultured using a substrate unsuitable for fermentation, suggesting that respiration impairment is a key factor in cell killing. This observation is in keeping with the notion that the respiratory chain is the main target for the action of sunlight in *Escherichia coli*.

**Key words:** Sunlight, survival, oxidative damage, scavengers, respiration, *Escherichia coli*.

1. Introduction

Solar radiation is a deleterious agent challenging the survival of bacteria in aquatic environments [1, 2]. The lethal effect of sunlight on bacteria has been recognized for a long time, but some basic aspects of the underlying mechanism remain unclear. It has been established that the lethal effect of solar radiation strongly depends on its ultraviolet component [3] and, since sunlight resistance increases when bacteria are exposed under nitrogen atmosphere, an important role was ascribed to the photodynamic action of the radiation [4]. Ultraviolet radiation could induce the formation of several reactive oxygen species (ROS) in bacteria and the intermediary responsible for cell killing during sunlight exposures has not been certainly identified. Moreover, there is a strong debate on the potential targets of ROS in irradiated bacteria and on how ROS or induced photoprodusts damage cells. The objective of the present study was to obtain information on these issues.

Artificial sources have been widely used to study the action of ultraviolet A radiation (UVA) on bacterial systems. The response of *Escherichia coli* to UVA involves defense systems which protect the cell against the harmful effects of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) [3], suggesting that these ROS are relevant in the mechanism of radiation induced damage. Recently, protein carbonylation [5] and inactivation of the respiratory chain [6] have been detected in *E. coli* after UVA irradiation. A radiation induced increase of O$_2$ and H$_2$O$_2$ generation by flavins associated to respiratory dehydrogenases and the generation of hydroxyl radical (HO•) from H$_2$O$_2$ by the Fenton reaction might be proposed to explain the above-mentioned observations. Nevertheless, H$_2$O$_2$ accumulation was undetectable after exposure of *Salmonella typhimurium* to artificial UVA at a fluence rate comparable to those expected during natural irradiations, and it was speculated that H$_2$O$_2$ generation...
could be an artifact produced by monochromatic UVA at high fluence rates [7]. Generation of singlet oxygen (1O2) from endogenous photo-sensitizers may occur in biological systems during UVA exposures [8], and a mechanism of membrane damage involving fatty acids oxidation by 1O2 has been proposed to explain the lethal effect of UVA on E. coli [9].

It would be of interest to study the roles played by HO• and 1O2 in the lethal effect exerted by solar radiation on bacteria. Since bacteria are unable to synthesize an enzyme capable to inactivating HO• or 1O2, this issue can not be addressed using mutant strains defective for defense systems. In this study, as an alternative approach, the radiation response of wild type cells of E. coli was assayed in the presence of compounds recognized as HO• and/or 1O2 scavengers. A comparison of the photo-protector effect between these compounds and oxygen depletion provided information on the extent of oxidative damage mediated by HO• and 1O2. Differences in reactivity between the scavengers toward these ROS provided information about the identity of the agent responsible for the loss of bacterial viability.

Bacteria contain a variety of chromophores absorbing radiation in the wavelength range corresponding to solar ultraviolet [3], and the products generated by oxidative damage to these targets have been studied by employing different biochemical techniques [5, 6, 10, 11]. As a whole, the available data suggest that several cellular components undergo potentially lethal damage during irradiation, but the identity of the target responsible for cell death remains an open question. Oxidative damage to lipids [9], DNA [10] or proteins of the respiratory chain [6] has been proposed as the event leading to the loss of bacterial viability in E. coli. In the present work, the relationship between the kinetics of cell death and production of energy through the respiratory chain was studied by culturing irradiated bacteria with different substrates. Complementing the information available from biochemical analysis, this physiological approach provided some clues of the mechanism of cell death.

2. Materials and Methods

2.1 Bacterial Strains and Culture Conditions

Escherichia coli K12 were cultured in L broth (10 g peptone, 5 g yeast extract, 5 g NaCl per liter) for 24 h at 37 °C with shaking. Cells in stationary phase were harvested by centrifugation (8,000 g, 8 min, 20 °C).

2.2 Sunlight Irradiation

Harvested bacteria were washed three times with sterile distilled water and suspended in 0.05 M sodium phosphate buffer, pH 7.2, supplied with 0.1 M mannitol (Mallinckrodt), or 0.02 M L-histidine (Sigma), when required. The optical density of the suspensions at 650 nm was adjusted to 0.1 (approximately 10⁸ colony forming units·mL⁻¹). Bacterial suspensions were prepared 45 min before the start of the irradiation, then transferred to the irradiation device and bubbled with air or nitrogen in the dark for 15 min before the start of the irradiation. Irradiations were performed with natural sunlight in order to mimic the conditions expected during natural exposures, avoiding eventual artifacts dependent on the characteristics of the incident radiation. The irradiation device and the protocol used, as well as the procedures for irradiance and sample temperature measurements, have been described previously [12]. The assays were performed on the roof of the laboratory (34°34′S, 58°30′W) on cloudless days at noon. Each irradiation condition was assayed at least three times in independent experiments. For dark controls, bacterial suspensions were submitted to the same procedure but under a dim light at the laboratory.

2.3 Enumeration Procedure and Data Analysis

At the onset of the experiments and at regular intervals during irradiations, samples were removed and diluted in decimal steps with sterile distilled water. Aliquots of suitable dilutions were spread on M9
minimal medium plates (1 g NH₄Cl, 0.5 g NaCl, 3 g KH₂PO₄, 6 g Na₂HPO₄, 0.2 g MgSO₄·7H₂O, 15 g agar per liter) with 0.011 M D-glucose (Sigma) or 0.022 M DL-sodium succinate (Merck) as the carbon source, and colonies were counted after incubation at 37 °C in the dark for 40 h. Survival curves were analyzed by using an adaptation of the target theory model, which assumed that in a bacterial population some cells lose viability due to a process characterized by a single-hit single-target kinetics and simultaneously damage accumulation inactivates other cells with a single-hit multiple-target kinetics [12, 13].

3. Results and Discussion

3.1 Effect of ROS Scavengers on the Sunlight Response of E. coli

A variety of compounds have been used as ROS scavengers in the study of oxidative stress induced by chemical agents and ionizing radiation, but some of them are unsuitable under the irradiation conditions used in this work. Catalase is quickly inactivated by sunlight exposure [14]. Cysteine can be oxidized to cystine by aeration of the bacterial suspensions and it was found to be inefficient as a HO• scavenger in vitro [15]. Other usually employed scavengers, including sodium pyruvate, thiourea, and the iron chelator o-phenanthroline were assayed in preliminary experiments (data not shown), but they conferred considerable absorbance to the irradiation medium in the wavelength range of 290-400 nm when added at the required concentrations. Additional effects of these compounds acting as a screen could contribute to photoprotection. Moreover, increased susceptibility induced by the addition of some of these agents was eventually observed, suggesting that they could act as photo-sensitizers.

Neither L-histidine nor mannitol present the disadvantages outlined above and they were chosen to study the effects of solar radiation. Several assays showed that both compounds reduce sunlight susceptibility of E. coli (Table 1). Representative survival curves obtained during simultaneous irradiations developed with nitrogen or air bubbling and in the presence or absence of L-histidine or mannitol are shown in Fig. 1. The addition of the scavengers reproduced for the most part the effect of a reduction of the oxygen concentration in the bacterial suspension. All curves seem linear except for an increase in the slope occurring when the imparted dose exceeded a quasi-threshold value, resembling the response of Salmonella enterica serovar typhimurium ATCC14028 irradiated under similar conditions [12]. Remarkably, at the concentrations used here scavengers produce similar changes in the sunlight response of E. coli indicating that most of ROS generated react with L-histidine and mannitol. Reported rate constants for the reactions of HO• with L-histidine and mannitol at neutral pH are 5 × 10⁹ M⁻¹s⁻¹ and 2.7 × 10⁹ M⁻¹s⁻¹, respectively, and that for the reaction of ¹O₂ with L-histidine is 3.2 × 10⁷ M⁻¹s⁻¹.

Table 1  Comparison of sunlight susceptibility of E. coli under different irradiation and post-irradiation conditions.

<table>
<thead>
<tr>
<th>Irradiation atmosphere</th>
<th>Added scavenger</th>
<th>Post-irradiation carbon source</th>
<th>Mean values for the estimated parametersa (MJ m⁻²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1/q                                      1/k      tₚ</td>
</tr>
<tr>
<td>air</td>
<td>none</td>
<td>glucose</td>
<td>2.91 (1.30) 0.34 (0.13) 3.64 (1.35)</td>
</tr>
<tr>
<td>nitrogen</td>
<td>none</td>
<td>glucose</td>
<td>6.44 (1.95) 0.44 (0.36) 6.83 (1.23)</td>
</tr>
<tr>
<td>air</td>
<td>mannitol</td>
<td>glucose</td>
<td>4.58 (0.24) 0.34 (0.02) 5.90 (1.99)</td>
</tr>
<tr>
<td>air</td>
<td>histidine</td>
<td>glucose</td>
<td>5.79 (0.92) 0.31 (0.04) 6.71 (0.57)</td>
</tr>
<tr>
<td>air</td>
<td>none</td>
<td>succinate</td>
<td>1.82 (0.26) 0.19 (0.03) 2.37 (0.19)</td>
</tr>
</tbody>
</table>

a: Parameters of the equation which describes the relationship among survival fraction and imparted dose according to the target theory model (equation (1) in reference [12]) were adjusted to experimental data obtained under different conditions; 1/q: fluence required for 1/e (37%) survival at the onset of the irradiation; 1/k: additional fluence required for 1/e survival after increment in the rate of cell death; tₚ: fluence required to start the increment in the rate of cell death.
Intermediaries and Targets of the Oxidative Stress Induced by Natural Sunlight in *Escherichia coli*

Fig. 1 Effects of oxygen depletion and addition of ROS scavengers on survival of *E. coli* K12 exposed to natural sunlight. Bacterial suspensions were irradiated in the absence of ROS scavengers while bubbled with air or nitrogen, or in the presence of histidine or mannitol while bubbled with air. Viability was measured using M9 glucose plates. Results of data analysis according to the target theory model [12] are shown as lines. Error bars represent standard deviations of counts. Irradiance (range of 200–1,100 nm) was 887–901 W·m⁻², and temperature of the samples was 25 °C.

However, a value lower than 10³ M⁻¹s⁻¹ was reported for the rate constant for reaction of ¹O₂ with mannitol [16]. Assuming that rate constants for these reactions in the cell are proportional to their *in vitro* values, and intracellular concentrations of the scavengers are proportional to their concentrations in the irradiation medium, the rates for *in vivo* reactions of L-histidine and mannitol with HO• could be in the same order. Conversely, the reaction of ¹O₂ with mannitol should be slower than that with L-histidine. Since ROS generated during sunlight exposures were scavenged by both L-histidine and mannitol with similar efficiency, HO• seems to be responsible for most of the oxidative damage observed under the conditions used.

Catalase inactivation is an early event during UVA exposure of *E. coli* [6] and *Salmonella typhimurium* [7]. This effect probably leaves H₂O₂ available for alternative reactions, like reduction to HO•. Since intracellular H₂O₂ concentration would depend on the balance between generation and consumption, HO• production could be accumulated. The absence of high H₂O₂ concentrations in the extracellular medium after UVA exposure [7] seems therefore compatible with a mechanism of cell death mediated by HO• production. Oxidative damage produced by ¹O₂ in *E. coli* exposed to UVA [9] was observed under experimental conditions which were different to those used in the present study. The irradiation of bacteria in exponentially growing phase and particularly the modification of the composition of membrane fatty acids could lead to a more favorable condition for the reactions mediated by ¹O₂.

It has been pointed out that the mechanisms of intracellular O₂⁻ and H₂O₂ production differ in site and rate from organism to organism, and different bacteria may undergo different amounts of O₂⁻ and H₂O₂ stress in the same environment [10]. The involvement of O₂⁻ and H₂O₂ generation in the lethal effect of UVA and the above-mentioned differences could be responsible for the variability in sunlight [17, 18] and UVA [19, 20] susceptibility found within phylogenetically related bacteria.

### 3.2 Substrate Availability and Survival of *E. coli* Exposed to Sunlight LE

Lipids, DNA and proteins react with HO• easily [3].
Given the preponderant role of this radical in the effect of sunlight, different mechanisms of damage could be proposed to explain the lethal effect of this radiation. Since polyunsaturated fatty acids are absent in membranes of *E. coli* [21], a mechanism of cell death involving oxidative damage to membrane lipids seems unlikely [10]. Single strand breaks and base modifications have been observed in *E. coli* DNA after UVA irradiation [22], suggesting that accumulation of DNA damage impairs DNA replication and cell division. Biochemical evidence also demonstrates the occurrence of oxidative damage to proteins, inactivation of the respiratory chain and energy depletion during exposure of *E. coli* to UVA [5, 6]. As an approach to test whether damage to the respiratory chain contributes to cell killing when *E. coli* is exposed to natural sunlight, surviving cells were counted using culture media supplied with glucose or succinate. Both compounds support growth of *E. coli* K12 on M9 minimal medium when it is used as the unique carbon and energy source. During exponential growth at 37 °C in this medium the growth rate for cells using succinate is approximately 2/3 of which observed for cells using glucose [23]. Glucose utilization allows *E. coli* to obtain energy by respiration and fermentation, but succinate provides energy by the respiration process only. The experimental approach takes advantage of this characteristic of *E. coli* metabolism. Typical results obtained with this protocol are shown in Fig. 2. Survival was considerably reduced when glucose was replaced by succinate as the unique carbon and energy source (Table 1). If DNA damage were the main even responsible for the loss of viability, this result would reach harmful levels without H2O2 indicate that during post-irradiation growth the efficiency of DNA repair changes due to the use of a different carbon source. It seems difficult to explain a detrimental effect of succinate on DNA repair and, in fact, the slow growth observed with this substrate could increase the time available for the repair process before DNA replication. Conversely, a mechanism of cell death involving damage to proteins of the respiratory chain and energy depletion could explain increased cell death observed when irradiated bacteria grew on succinate, because under this condition fermentation was not available as an alternative pathway to obtain energy. The results

**Fig. 2** Effect of post-irradiation growth with different carbon sources on survival of *E. coli* K12 exposed to natural sunlight. A bacterial suspension was irradiated in the absence of ROS scavengers while bubbled with air. Viability was measured by plating irradiated bacteria on M9 plates supplied with D-glucose or DL-sodium succinate. Results of data analysis according to the target theory model [12] are shown as lines. Error bars represent standard deviations of counts. Irradiance was 901-908 W·m⁻², and temperature of the sample was 24 °C.
shown in Fig. 2 are therefore in keeping with the notion that energy deficiency is the main factor leading to the loss of viability in irradiated bacteria, as proposed by Bosshard and co-workers [6].

Increased survival has been reported when *E. coli* cells exposed to simulated sunlight were cultured under anaerobic conditions, and oxidative stress produced during post-irradiation incubation was proposed as an explanation for this observation [24]. In addition to avoid the eventual post-irradiation oxidative stress, the absence of O₂ could induce the adaptation of irradiated bacteria to obtain energy by alternative pathways thereby alleviating the effect of damage to the respiratory chain.

4. Conclusion

Under irradiation conditions resembling those expected during natural exposures HO⁻ production seems to be responsible for most of the lethal effect of sunlight in *E. coli*. Impairment of respiration probably plays an important role in the mechanism leading to the loss of viability. Techniques for water disinfection using solar radiation usually involve procedures comparable with the irradiations performed in this study [25]. The influence of substrate availability on survival described in the present study highlights the importance of post-irradiation culture conditions in the evaluation of the efficiency of these techniques.

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References


Prevention of Carbon Tetrachloride (CCl₄)-Induced Liver Damage in Guinea Pigs by Cyphostemma digitatum

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Abstract: Cyphostemma digitatum (Vitaceae) is a perennial, climbing, succulent undershrub with compound fleshy leaves and tendrils. The plant is used mainly as a food flavoring, but it is also a main constituent of traditional Yemeni soup (Marak). Besides that, it has been described to be used as a medicinal plant. The aim of this work was to study the hepatoprotective effect of the aqueous extract of C. digitatum against CCl₄-induced liver injury in Guinea pigs. Animals were divided into four groups. Group I, served as normal control. Group II received 2 mL CCl₄/kg b.w. diluted with olive oil, at 1:1 ratio on day 11. Group III (test group) was pre-treated orally with 100 mg/kg b.w. aqueous leaves extract of C. digitatum for 10 days followed by subcutaneous injection of CCl₄ (2 mL/kg b.w.), once on day 11. Group IV were orally given Liv-52 (100 mg/kg b.w.) once daily for 10 days followed by subcutaneous injection of CCl₄. Our results show that, the activity of serum hepatic enzymes (alanine aminotranferase (ALT), aspartate aminotranferase (AST), and alkaline phosphatase (ALP)) were significantly elevated in Guinea pigs treated with CCl₄, while both the C. digitatum extract and Liv-52 reduced significantly these enzymes activity. Also, the levels of glucose, urea, cholesterol and triglycerides were decreased when compared with intoxicated control. Histopathological examination of intoxicated animals showed fatty changes, inflammation and necrosis indicating liver damage, while the animals received C. digitatum or Liv-52 showed less pathological effects or normal liver when compared to animals treated with CCl₄ alone. Biochemical and histological results confirm the hepatoprotective effect of aqueous extract of C. digitatum.

Key words: Cyphostemma digitatum extract, CCl₄, liver damage, Guinea pig.

1. Introduction

The search for new drugs and formulations that are safe, affordable and effective against both early and late stages of the disease is recommended [1-3]. Plant-derived substances/drugs have recently become of great interest owing to their versatile applications. In particular, herbal medicine is as old as the history of humankind [4, 5]. This is traceable to the growing belief by consumers that herbal remedies are safe and effective. Indeed, the effectiveness of some medicinal herbs in the treatment of disease has been validated through research and clinical studies [6]. Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions [7].

Several plant constituents have proven to show free radical scavenging or antioxidant activity [8-10]. Flavonoids and other phenolic compounds of plant origin have been reported as scavengers and inhibitors of lipid peroxidation [11, 12].

C. digitatum (Vitaceae) is a perennial, climbing, and succulent undershrub with compound fleshy leaves and tendrils. The leaves are petiolate, digitately 3-5 foliolate; leaflets are ovate and dentate [13]. C. digitatum usually occurs between 1,400 and 2,500 m a.s.l., often on cliffs and with preference for shaded stony places such as gullies and terraces walls [14]. The
leaves and fleshy young stem branches are used in dried form after processing. The plant is used mainly as a food flavoring, but it is also a main constituent of traditional Yemeni soup (Marak). Besides that, it has been described to be used as a medication for gastroenteritis, fatigue, vomiting, and headache, against malaria, and for general health support [13].

Carbon tetrachloride (CCl₄) is a highly toxic chemical agent, widely used to elicit experimental liver damage. The metabolic disorders and metabolic syndromes of CCl₄ induced hepatotoxicity have been investigated in literature [15-18]. Acute and chronic liver diseases constitute a global concern and medical treatments for these diseases are often difficult to handle and have limited efficiency [19]. Therefore, there has been considerable interest in role of complementary and alternative medicines for the treatment of liver disease. Developing therapeutically effective agents from natural products may reduce the risk of toxicity when the drug is used clinically [20].

The aim of the present study was to study the hepatoprotective effects of the aqueous leaves extract of *Cyphostemma digitatum* against CCl₄-induced acute liver injury in Guinea pigs.

2. Materials and Methods

2.1 Plant Materials

The leaves of *C. digitatum* were collected in April 2010 from Al-Siany district, Ibb governorate, Yemen. The plant was authenticated by comparison with reference specimens preserved at the Herbarium of Biological Department, Ibb University. Voucher specimens were kept in the Herbarium for future references.

2.2 Preparation of Aqueous Extract

The leaves were washed, cut into small pieces, shade dried for five days, and then dried overnight in an oven. The dried leaves (200 g) were boiled for 30 min with distilled water (2,000 mL). The resulting water extract was filtered and subsequently concentrated with a water bath (90 °C) until it became creamy, and was then dried in an oven (60 °C) that finally gave 30 g (15% of initial amount) of powder [21]. The dried extracts were dissolved in water and administrated orally when experiments were performed.

2.3 Chemicals and Kits

All the drugs and chemicals used in the study were of analytical grade. Carbon tetrachloride was obtained from Merck Limited, India. Liv-52 was obtained from Himalaya Drug Company, Bangalore, India. Diagnostic kits for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea, glucose, total cholesterol (TC) and triacylglycerol (TAG) were purchased commercially from Spinreact (Spain).

2.4 Experimental Animals

Twenty adult male guinea pigs (350-700 g) were obtained from the animal house of Biology Department and kept for one week on a commercial diet in environmentally controlled conditions (25 ± 5 °C, 55 ± 5% humidity and 12 h light-dark cycle) with free access to diet and water *ad libitum*.

2.5 Experimental Protocol

The experimental animals were allocated into four groups, each containing five animals. In group I, (control) animals were received vehicle. In group II, animals received subcutaneous injection of 2 mL CCl₄/kg body weight (b.w.) diluted with olive oil, at 1:1 ratio on day 11. In group III, animals were orally pre-treated with 100 mg/kg b.w. of aqueous leave extract of *C. digitatum* for 10 days followed by subcutaneous injection of CCl₄ (2 mL/kg b.w.) on day 11. In group IV, animals were orally pre-treated with 100 mg/kg b.w. of Liv-52 for 10 days followed by subcutaneous injection of CCl₄ on day 11.

2.6 Preparation of Samples

Twenty-four hours after the administration of CCl₄, the animals of each group were anaesthetized with
ether, and blood was collected directly from the portal vein. The blood sample of each animal was separated to obtain serum for biochemical analysis [22, 23].

2.7 Biochemical Studies

Serum AST, ALT and ALP were determined kinetically using Spinreact Diagnostics kits (Spain). Urea, glucose, cholesterol and triacylglycerol were evaluated colorimetrically in blood using spectrophotometric diagnostic kits of Spinreact (Spain).

2.8 Histopathological Examination

Control and experimental animals were put under light ether anaesthesia, dissected as quickly as possible, and then livers were removed. Small pieces were fixed in 10% neutral formalin for 24 h, then washed by the running tap water, and stored in 70% ethyl alcohol, until further processing. Blocks of about 5 × 5 mm size were dehydrated, cleared and embedded in paraffin wax. Paraffin sections of five microns thickness were cut using rotary microtome (Leica, Germany) and sections were flattened on the surface of warm water using a water bath. Slides were then dried on a hot plate for 30 minutes and stored in the incubator at 37 ºC and stained with Ehrlich’s haematoxylin and counter-stained with eosin. Then mounted in Canada balsam, labeled and became ready for microscopic examination.

2.9 Statistical Analysis

Results of the biochemical estimations are reported as mean ± standard deviation (S.D.). Total variation, present in a set of data was estimated by one-way analysis of variance (ANOVA). Differences with a P-value < 0.05 were considered as statistically significant [24].

3. Results

3.1 Biochemical Results

The results of the present study show that, CCl₄ intoxication causes a significant (P < 0.05) elevation of AST, ALT, and ALP by 2.45, 3.7 and 3.4 times, respectively when compared to control animals. On one hand, the pretreatment of animals with aqueous leaves extract of C. digitatum significantly (P < 0.05) decreases AST, ALT, and ALP by 2.29, 3.15 and 6.04 times respectively compared to CCl₄ group. Similar results were also obtained with Liv-52 + CCl₄ group (Table 1). These results show the development of severe hepatic injuries in this group as compared to animals pretreated with leaves extract of C. digitatum or those that were pre-treated with 100 mg/kg b.w. of Liv-52. Also, treatment of animals with CCl₄ only induced a significant increase (P < 0.05) in glucose and urea by 1.78 and 1.41 times respectively compared to control group (Table 2). In addition, orally pretreatment of animals with C. digitatum or Liv-52 reduces the levels of glucose significantly (P < 0.05) by 1.64 and 2.08 respectively in both groups with respect to CCl₄ group alone. On the other hand, the levels of urea were decreased in C. digitatum group compared to CCl₄ group, but this decrease was not significant (P > 0.05), while this decrease was significantly (P < 0.05) in Liv-52 group by 2.10 fold when compared to CCl₄ group (Table 2). The level of serum cholesterol in control group was found to be 1.16 ± 0.41 mmol/L and the cholesterol level was increased significantly (P < 0.05) in the animals treated with CCl₄ by about 2.21 folds. C. digitatum treatment caused significant fall (P < 0.05) in this group to 1.61 ± 0.17 mmol/L (Table 2). The administration of CCl₄ only induces significant increase (P < 0.05) of triglycerides by 3.9 folds compared to control value (Table 2). The pretreatment of animals with extract of C. digitatum was not improving the levels of triglycerides.

3.2 Histopathological Results

Animals in the normal group showed normal hepatic lobular architecture with central veins and radiating heptatics cords (Fig. 1A). CCl₄ induces various pathological alterations in liver of Guinea pigs. These
Table 1  Effect of aqueous leave extract of *C. digitatum* on the activity of plasma liver enzymes in Guinea pigs treated with CCl4.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Groups</th>
<th>Control</th>
<th>CCl4</th>
<th>C. digitatum + CCl4</th>
<th>Liv-52 + CCl4</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST</td>
<td></td>
<td>37.83 ± 5.35</td>
<td>92.66 ± 17.14</td>
<td>40.4 ± 1.55 b</td>
<td>66 ± 13.52 b</td>
</tr>
<tr>
<td>ALT</td>
<td></td>
<td>50.6 ± 11.26</td>
<td>187.6 ± 20.69</td>
<td>59.6 ± 2.47 b</td>
<td>48.66 ± 3.05 b</td>
</tr>
<tr>
<td>ALP</td>
<td></td>
<td>120 ± 19.12</td>
<td>287.5 ± 46.79</td>
<td>47.56 ± 1.69 b</td>
<td>51.33 ± 10.1 b</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D., n = 5. Values marked with asterisks differ significantly from control value: *P* < 0.05; those marked with letter differ significantly from CCl4 group: *P* < 0.05.

Table 2  Effect of aqueous leave extract of *C. digitatum* on plasma glucose, urea, triglycerides and cholesterol in guinea pigs treated with CCl4.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Groups</th>
<th>Control</th>
<th>CCl4</th>
<th>C. digitatum + CCl4</th>
<th>Liv-52 + CCl4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td></td>
<td>5.61 ± 0.34</td>
<td>9.96 ± 0.76</td>
<td>6.08 ± 2.20 b</td>
<td>4.8 ± 0.11 b</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td>7.6 ± 0.479</td>
<td>10.69 ± 0.9</td>
<td>8.18 ± 0.72</td>
<td>5.077 ± 2.35 b</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td>0.72 ± 0.16</td>
<td>2.8 ± 0.57</td>
<td>2.47 ± 0.26</td>
<td>0.49 ± 0.03 b</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
<td>1.16 ± 0.41</td>
<td>2.56 ± 0.12</td>
<td>1.61 ± 0.17 b</td>
<td>1.0 ± 0.07 b</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D., n = 5. Values marked with asterisks differ significantly from control value: *P* < 0.05; those marked with letter differ significantly from CCl4 group: *P* < 0.05.

alterations were characterized by dilation of central vein with marked hemorrhage. Moreover, dilation of blood sinosids, necrosis and cytoplasmic vaculaization of hepatocytes were observed in the liver sections of CCl4 group compared with control (Fig. 1B). In the *C. digitatum* group, liver showed less histopathological changes when compared to CCl4 group (Fig. 1C). On the other hand, Liv-52 also showed hepatoprotective against CCl4-induced liver damage when compared to CCl4 group alone, but it was not good as that observed with *C. digitatum* group (Fig. 1D).

4. Discussions

The liver is major organ responsible for the metabolism of drugs and toxic chemicals and therefore is the primary target organ for nearly all toxic chemicals [25, 26]. CCl4 is a frequently used model substance for hepatotoxicity studies [27]. It has been well established that CCl4 is metabolized in the liver to the highly reactive trichloromethyl radical and this free radical leads to auto-oxidation of the fatty acids present in the cytoplasmic membrane phospholipids and causes functional and morphological changes in the cell membrane [28]. The results of the present study showed that, CCl4 intoxication causes a significant elevation of liver enzymes when compared to control animals. This elevation could potentially be attributed to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage [29]. Our results were in consistence with previous investigations [30, 31].

The rise in the enzyme AST is usually accompanied by an elevation in the levels of ALT, which plays a vital role in the conversion of amino acids to keto acids [32]. Our results showed that pretreatment of Guinea pigs with aqueous leaves extract of *C. digitatum* exhibited a significant reduction in the levels of liver enzymes. This is evidenced in the marked decrease in these enzymes relative to the group treated with CCl4 alone. These results indicated that the extract of *C. digitatum* was able to protect the membrane integrity against CCl4-induced leakage of marker enzymes into the circulation [6]. Our results were in agreement with Al-Duais et al. [33] who found that *C. digitatum* contains high levels of vitamin C, vitamin E, carotenoids, and tocotrienols. It has been well established
Prevention of Carbon Tetrachloride (CCl₄)-Induced Liver Damage in Guinea Pigs by Cyphostemma digitatum

Fig. 1 The effect of C. digitatum against CCl₄-induced hepatotoxicity in Guinea pigs. Liver sections were stained with H & E: (A) normal; (B) CCl₄ (2ml/kg b.w.) treated animals; (C) C. digitatum (100 mg/kg b.w.) + CCl₄; (D) Liv-52 (100 mg/kg b.w.) + CCl₄, magnification 400 X. (H) hepatocytes, (CV) central vein, (N) nucleus, (S) sinusoidal space, (BH) binucleated hepatocytes, (h) hemorrhage, (CN) condensed nucleus, and (FN) fragmented nucleus.

that vitamin C, vitamin E, and β-carotene all displayed antioxidant activity and thus provided cellular defense against reactive oxygen species, which could damage the DNA [34]. These vitamins may scavenge free radicals generated by CCl₄ and reduces lipid peroxidation. Additionally, cooperative interactions between vitamin E and vitamin C in protecting against lipid peroxidation in liposomes have been examined [35]. Liv-52 is widely used in treatment of liver diseases of varying origins. It enhances tocopherol levels, which inhibits lipid peroxidation; scavenges free radicals [36]. In the present study, Liv-52 caused a significant decrease in serum enzymes activity induced by CCl₄ in Guinea pigs. These results were in agreement with previous investigation [37, 38]. Also, CCl₄ induced significant elevation of cholesterol and triglycerides. While, pretreatment of animals with C. digitatum reduces significantly cholesterol and triglyceride levels suggesting that C. digitatum could have protective effect on the cardiovascular system [39]. The hypotriglyceridemic effect may be through its effect on increasing the activity of lipase [40]. According to Seo et al. [41], β-carotene reduced the elevation of cholesterol and triglycerides of diabetic rats. The elevation of glucose level in the present study could be attributed to destruction of hepatocytes induced by CCl₄ intoxication [42] or decreasing of glycogen contents in hepatocytes [43]. Pretreatment of animals with aqueous leaves extract of C. digitatum improves serum glucose level. Histopathologically, the protective effectiveness of C. digitatum and Liv-52 in the prevention of CCl₄-induced liver damage was observed. In CCl₄ group, hepatocellular necrosis and cytoplasmic vacuilaization were marked in the
histopathological examination of all sections. However, in the *C. digitatum* and Liv-52 groups little or mild histopathological alterations were observed. Thus, the finding of this study shows that the administration of an aqueous leaves extract of *C. digitatum* appeared to protect the liver of Guinea pigs from CCl₄-induced acute oxidative stress possibly through antioxidant activities [13]. It has been established that reactive oxygen species are involved in inflammation [44], and the protective action of *C. digitatum* extract against CCl₄-induced hepatic damage could involve mechanisms related to scavenging activity. The present investigation is important in presenting data suggesting considerable promise for the *C. digitatum* as a protective agent in CCl₄-induced damage on liver.

### 5. Conclusion

In conclusion, our results indicated that the pretreatment of guinea pigs with *C. digitatum* leaves extracts ameliorate CCl₄-induced liver damage significantly. Hence the leaves extracts of *C. digitatum* might be effective hepatoprotectors in the diets of patients with hepatopathies. Our data warrant further investigation of this extract as a potential treatment in other models of hepatic disorder.

### Acknowledgments

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### References

Prevention of Carbon Tetrachloride (CCl₄)-Induced Liver Damage in Guinea Pigs by Cyphostemma digitatum


The Potency of *Sargassum duplicatum* Bory Extract on Inflammatory Bowel Disease Therapy in *Rattus norvegicus*

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Abstract: Inflammatory Bowel Disease (IBD) was a chronic inflammatory disease of the digestive tract especially in small and large intestines that induced by indomethacin. Potency of ethanol and ethyl acetate extract from brown seaweed (*Sargassum duplicatum* Bory) against indomethacin induced jejunum damage was evaluated in *Rattus norvegicus*. Control rats induced by corn oil orally. IBD rats induced by indomethacin of 15 mg/kg body weight (bw) orally and incubated for 7 days. Therapy rats were treated orally by brown seaweed extract of 100 mg/kg bw respectively for seven days. Based on phytochemistry test, *Sargassum duplicatum* Bory extract contains flavonoids, phlorotannin, and alkaloid. The result of preparative Thin Layer Chromatography (TLC) and Infra Red (IR) spectrum of extract spots showed the same result (function group similarity) with gallic acid standard as polyphenol. *Sargassum duplicatum* Bory extract decreased Malondialdehid (MDA) level (54.20%) significantly using Thiobarbituric Acid (TBA) assay, repaired ZO-1 and occludin protein expressions by immunohistochemistry and repaired jejunum damage by histological observation.

Key words: Indomethacin, IBD, *Sargassum duplicatum* bory extract, MDA, ZO-1, occludin, histology of jejunum.

1. Introduction

Inflammatory Bowel Disease (IBD) was a chronic inflammatory disease that attacks the digestive tract, especially small intestine and colon. Common symptoms of IBD are diarrhea, abdominal pain, gastrointestinal bleeding and damage the digestive tract [1]. Based on prospective study in four hospitals in Jakarta Indonesia from year 1999 to 2009, there were 213 patients with IBD from 386 total cases using endoscopic examination [2]. Patients with IBD in United States from 1997 to 2007 amounted to 213 thousand people while 43 thousand people in Britain [3].

In general, IBD is caused by bacteria or viruses infection in the digestive tract [4]. Some researches suggested that the IBD caused by side effect application of non-steroidal anti-inflammatory drug (NSAID), such as indomethacin [5]. Indomethacin inhibit the cyclooxygenase (COX) 1, PGE₂ synthesis and production of mucus that protect the intestinal mucosa against to bacterial and viral infection [4, 6]. Indomethacin was proved to increase the production of reactive oxygen species (ROS), the radical O₂⁻, OH⁻ and H₂O₂ derived from the leakage of electrons from the electron transport chain specific, indomethacin metabolite of oxidation process desmetildeschloro-benzoyl-indomethacin (DMBI) becomes iminoquinon and activation of neutrophils and macrophages (a process of phagocytosis) [6-8]. Indomethacin increased levels of malondialdehyde (MDA) and decreased activity of the superoxide...
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Dismutase (SOD), glutathione (GSH) peroxidase and glutathione reductase, which are marker of oxidative stress [9].

ROS produced by indomethacin and reactive metabolites that can stimulate inflammatory process followed by tissue damage to the small intestine. This will lead to changes in the structure and weaken the defense system of cells especially tight junctions. Tight junctions are composed of three transmembrane proteins: occludin, claudin and junctional adhesion molecules (Jams). Transmembrane protein are associated with adapter proteins zone occludens 1 (ZO-1), ZO-2 and ZO-3 [10, 11].

Conventionally, in IBD cases performed by administration of cortisone (steroids), anti-inflammatory, immune system suppressors and antibiotics. In addition, there are herbal therapies like ethanol and methanol extracts of *Flos lonicerae* (tea) and methanol extract of *Rhizoma bletillae* (orchid). They are rich in polyphenols as antioxidants [12, 13].

Antioxidant activity contained in brown seaweed (*Sargassum* sp.) is polyphenol compounds (flavonoids and phlorotanin) and fukosantin [14, 15]. The content of antioxidant polyphenols (flavonoids) 85% ethanol extract of *Sargassum duplicatum* Bory with a dose of 100 mg/kg body weight of rats proved able to reduce MDA levels that indirectly reflect the decreased levels of free radicals [16]. Phlorotanin crude ethanol extract and ethyl acetate *Sargassum* sp. in addition have antioxidant and anti-allergic activity [15].

Based on the above, then the research will be assessed the potency of ethanol and ethyl acetate extract from brown seaweed (*S. duplicatum* Bory) as scavenger free radicals in IBD animal which then used as an alternative therapy of IBD.

2. Materials and Methods

2.1 Preparation of Animals

This study used twenty-four male rats (*Rattus norvegicus*), 12 weeks old, wistar strain, and 125-175 g weighed. Rats were divided into three groups: control group (healthy), IBD group (exposed to indomethacin) and therapy group. Experimental animals were adapted for a week in animal box keeping. Control group: induced by corn oil orally. IBD group: exposed to indomethacin of 15 mg/kg bw orally then incubated for seven days. Therapy group: exposed to indomethacin of 15 mg/kg bw orally and then treated with brown seaweed extract of 100 mg/kg bw for 7 days. On ninth day, jejunum rat was dissected and retrieved. All animals were bred and maintained in our animal facility in University of Brawijaya, Indonesia, and treated in accordance with the guide for animal experimentation of Ethical Committee in the Care and Use Animals, University of Brawijaya.

2.2 Preparation of Ethanol and Ethyl Acetate Extract from Brown Seaweed

Brown seaweed was cleaned and cut into small size then wind dried until contain between 20-30% of moisture content. Brown seaweed was weighed as much as 116 g and extracted by maceration using 1.5 L, 85% of ethanol. Maceration performed for two days. The extract was then filtered and the filtrate was concentrated by rotary vacuum evaporator at 40 °C (± two hours). Extract was concentrated and then washed with each 100 mL of chloroform three times and the upper layer (non-lipid fraction) was extracted with 250 mL of ethyl acetate. Ethyl acetate fraction was taken and dried with N₂ gas to obtain extracts with a constant weight.

2.3 Phytochemistry Test of Extract

Flavonoid test was done by adding of NaOH 10% to solution extracts and positive results is shown by the yellow color and becomes translucent or turbid colorless after the addition of dilute HCl. Phlorotanin test performed with the addition of FeCl₃ and into the extract solution. Positive results were indicated by a blue-black color. Alkaloid test was done by adding 10 mL of NH₃ (ammonia) 0.05 M and chloroform (CHCl₃) into 4 g of extract solution. Solution was then mixed
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with 1 mL, 2 M H₂SO₄, shaken until formed two layers. Sulfuric acid layer was taken and added Wagner reagents. Positive alkaloid results if brown precipitate formed in solution. Terpenoids test performed using Salkowski test: as much as 0.5 g of extract plus 2 mL of CHCl₃, then add 2 mL of concentrated H₂SO₄. Positive results terpenoids, if red-brown color formed at the interface of two layers of solution.

2.4 Thin Layer Chromatography (TLC)

Brown seaweed extract isolated using thin layer chromatography (TLC) to separate a mixture of compounds contained in the extract. Mobile phase (eluent) was methanol: ethyl acetate: diethyl ether (10:5:2). The spots on the silica plate then viewed with UV light at a wavelength of 254 nm and 366 nm. Each spot of its Rf value was calculated using the formula:

\[ Rf = \frac{\text{distance of spot from the initial movement}}{\text{distance of eluent from the initial movement}} \]

2.5 Infra Red (IR) Spectrophotometric

IR spectrophotometric analysis aims to identify functional groups of compounds contained in brown seaweed extract. Each spots on the silica plate is scraped and analyzed by IR spectrophotometer using KBr (Potassium Bromide) pellets.

2.6 Histology of Small Intestine (Jejunum)

Histology technique was used for structure analysis of small intestine (jejunum). The section was stained with hematoxylin-eosin (HE) and then examined with light microscopy.

2.7 Determination of Malondialdehyde (MDA) Level

The determination of MDA level using of 100 μL jejunum homogenate, 550 μL of distilled water, 100 μL of TCA 10% (trichloro acetic acid), 250 μL of 1 M HCl and 100 μL of 0.8% TBA (2-thiobarbituric acid). The aliquots were homogenized and centrifuged at 500 rpm for 10 min. Supernatant was taken and incubated in a water bath 100 °C for 30 mins. Supernatant left in room temperature and read using spectrophotometer at 532 nm. MDA level was calculated from linear regression equation (standard curve) prepared using TBA standard.

2.8 Immunohistochemistry

Prepare of small intestine jejunum washed with PBS (Phosphate Buffer Saline) pH 7.4 and dropped with 3% H₂O₂ for 20 mins, washed with PBS pH 7.4, blocked with 5% BSA (Bovine Serum Albumin) for 30 min, washed with PBS pH 7.4, incubated with goat anti-ZO-1 primary antibody during overnight at 4 °C washed with PBS pH 7.4. Incubated using mouse anti goat IgG biotin labelled secondary antibody for one hour at room temperature, washed with PBS pH 7.4. Dropped with SA-HRP (Strep-Avidin Horse Radish Peroxidase), incubated for 10 min and washed with PBS pH 7.4, dropped with DAB (Diamano Benzidine) and incubated for 10 min and washed with aquades, counterstained with Mayer Hemotoxylin for 10 min. Prepare washed with flow water and then rinsed with aquades and dried. Mounted preparate with entellan and closed with cover glass. Positive results of ZO-1 expression showed if there was brown color on the preparate that examined with light microscopy. The same method was used to analyze the occludin expression using goat anti-occludin primary antibody and mouse anti goat IgG biotin labelled secondary antibody [17].

2.9 Statistical Analysis

Data were analysed using anlysis of variance (ANOVA) Kruskal Wallis, using software programe SPSS for Windows version 13.0.

3. Results

3.1 Results of Phytochemistry Tests, Thin Layer Chromatography (TLC) and Infrared Spectrum of Brown Seaweed (*S. duplicatum* Bory) Extract

Phytochemistry tests result of brown seaweed (*S. duplicatum Bory) extract showed positive result for flavonoids, phlorotanin, and alkaloids, but negative for terpenoids. They are characterized by distinctive color...
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changes in each treatment. Flavonoid test of brown seaweed extract showed positive results indicated yellow color after the addition of NaOH 10% and become translucent or turbid colorless after the addition of dilute HCl. Phlorotanins test of brown seaweed extract showed positive results indicated by a blue-black color after the addition of FeCl₃. Alkaloids test of brown seaweed extract showed positive results indicated by brown precipitate formed in solution after the addition of Wagner’s reagent. Terpenoids test of brown seaweed extract showed negative result indicated by no formation of red-brown color formed at the interface of two layers of solution after the addition of Salkowski’s test.

Brown seaweed extract separated and isolated of compounds by using the method of Thin Layer Chromatography (TLC). Eluent/mobile phase used a mixture of methanol:ethyl acetate:diethyl ether (10:5:2). Stationary phase was silica plate. TLC results showed the separation of the three spots are visible under UV light at a wavelength of 366 nm and 254 nm. Rf value of the three spots in a consecutive at (A, B, C) 0.50, 0.59 and 0.84 (figure and Rf calculations are shown in Fig. 1 and Table 1).

IR spectra of gallic acid (Fig. 2) showed the presence of absorption bands at wave numbers of 3,402.20 cm⁻¹ which indicate

The standard of gallic acid containing O-H (phenol, alcohol), C-H (aliphatic, aromatic), C=C (aromatic, alkena), and C-O.

IR spectra of spot A (Fig. 3) showed the absorption bands at wave numbers of 3,402.20 cm⁻¹ which indicate

![Fig. 1  TLC of brown seaweed extract using eluent (mobile phase) methanol : ethyl acetate : diethyl ether (10:5:2).](image)

<table>
<thead>
<tr>
<th>Spot</th>
<th>Distance of spot from the initial movement</th>
<th>Rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4 cm</td>
<td>8 cm</td>
</tr>
<tr>
<td>B</td>
<td>4.7 cm</td>
<td>8 cm</td>
</tr>
<tr>
<td>C</td>
<td>6.75 cm</td>
<td>8 cm</td>
</tr>
</tbody>
</table>

![Table 1  The Rf value of each spot brown seaweed extract by TLC method.](image)

Fig. 2  IR spectra of gallic acid standard [13].
The Potency of Sargassum duplicatum Bory Extract on Inflammatory Bowel Disease Therapy in Rattus norvegicus

Fig. 3  IR spectra of spot A TLC of brown seaweed extract.

Table 2  Interpretation of infrared absorption (IR) samples and standard.

<table>
<thead>
<tr>
<th>No.</th>
<th>Spot A Wave length (cm⁻¹)</th>
<th>Spot B Wave length (cm⁻¹)</th>
<th>Spot C Wave length (cm⁻¹)</th>
<th>Gallic acid standard [13]</th>
<th>Ref. [17] Wave length (cm⁻¹)</th>
<th>Interpretation</th>
<th>Vibration type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3,402.20</td>
<td>3,561.31</td>
<td>2,960.40</td>
<td>3,473.56</td>
<td>2,931.51</td>
<td>3,407.05</td>
<td>2,865.75</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2,850-3,000</td>
<td>C-H aliphatic</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1,705-1,750</td>
<td>C=O aldehyde, ketone, carboxylic acid and ester</td>
</tr>
<tr>
<td>4</td>
<td>1,641.31</td>
<td>1,657.70</td>
<td>1,639.70</td>
<td>1,511.23</td>
<td>1,441.69</td>
<td>1,475-1,600</td>
<td>C=O aromatic</td>
</tr>
<tr>
<td>5</td>
<td>1,511.23</td>
<td>1,506.30</td>
<td>1,509.58</td>
<td>1,420.99</td>
<td>1,184.21</td>
<td>1,170-1,465</td>
<td>C-H aliphatic</td>
</tr>
<tr>
<td>6</td>
<td>1,441.69</td>
<td>-</td>
<td>-</td>
<td>1,420.99</td>
<td>1,184.21</td>
<td>1,000-1,300</td>
<td>C-O (alcohol, ether, ester, carboxylic acid)</td>
</tr>
<tr>
<td>7</td>
<td>1,223.75</td>
<td>1,195.78</td>
<td>1,153.35</td>
<td>1,153.78</td>
<td>1,097.08</td>
<td>777.58</td>
<td>C-H aromatic</td>
</tr>
<tr>
<td>8</td>
<td>805.23</td>
<td>795.58</td>
<td>815.83</td>
<td>763.13</td>
<td>690-900</td>
<td>763.13</td>
<td>O=O aromatic</td>
</tr>
</tbody>
</table>

the presence of OH group. Absorption at 1,641.31 cm⁻¹ probably represents alkene C=C. Absorption at 1,511.23 cm⁻¹ represents aromatic C=C and at 805.23 cm⁻¹ is aromatic C-H. Absorption at 1,441.69 cm⁻¹ was aliphatic C-H. Spot A is also absorbed at 1,223.75; 1,195.78; 1,152.39 and 1,016.42 that represent C-O bond. Based on these IR data, in general, isolates A spot containing compounds with OH group, alkene C=C, aromatic C=C, aromatic C-H, aliphatic C-H and C-O bond.

IR spectra of spot B (Fig. 4) showed the absorption bands at wave numbers of 3,561.31 dan 3,473.56 cm⁻¹ which indicate the presence of OH group. Absorption at 1,657.70 cm⁻¹ probably represents alkene C=C. Absorption at 1,506.30 cm⁻¹ represents aromatic C=C and at 795.58 cm⁻¹ is aromatic C-H. Spot B also absorbs at 1,184.21; 1,113.81 and 1,097.08 cm⁻¹ represents C-O bond. Based on these IR data, in general, isolates B spot containing compounds with OH group, alkene C=C, aromatic C=C, aromatic C-H, and C-O bond.

IR spectra of spot C (Fig. 5) showed the absorption bands at wave numbers of 1,153.35; 1,096.46
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Fig. 4  IR spectra of spot B TLC of brown seaweed extract.

Fig. 5  IR spectra of spot C TLC of brown seaweed extract.

and 1,068.49 cm⁻¹ which indicate the presence of C-O bond. Spot C also absorb at 815.53 cm⁻¹ that represents aromatic C-H. Based on these IR data, in general, isolates B containing compounds with C-O bond and aromatic C-H.

IR spectra of three spots from TLC results then compared with standard spectra of gallic acid and indicating that brown seaweed (S. duplicatum Bory) extract mainly contain OH group, alkene C=,C, aromatic C=, aromatic C-H, aliphatic C-H and C-O bond as phenolic/polyphenols (flavonoids and phlorotanin) and alkaloids compounds.

3.2 Results of Malondialdehyde (MDA) Level of Rat Jejunum

MDA was the end product of lipid peroxidation membrane by ROS, so that measurement of MDA level indirectly reflect level of free radicals. The results
showed that the average MDA level of jejunum exposed to indomethacin group, 4.596 ± 0.429 (μg/mL). This value was higher than negative control group, 1.839 ± 0.067 (μg/mL) and therapy group, 2.105 ± 0.035 (μg/mL) (Fig. 6 and Table 3).

MDA level of control and IBD groups showed (Fig. 6 and Table 3) significantly different (P < 0.05). It showed that MDA level of IBD group significantly increased to the control. After treatment with brown seaweed extract, MDA level of therapy group significantly decreased but cannot reach the same level with control group.

3.3 Results of Histological Picture of Rat Jejunum

Histological picture was used to determine the level of damage and repairment on tissue. Fig. 7 presented the small intestine jejunum picture from negative control, IBD (exposed to indomethacin and then incubated for seven days) and therapy (exposed to indomethacin and then treated with extract of brown seaweed).

Fig. 7 showed the mucosal jejunum of negative control group (a) was still good. It has long and compact villi structure. But in IBD rat (b), there was damage to the mucosa, the villi become shorter, broken and there was hollow space. After given with ethanol and ethyl acetate extract from brown seaweed (S. duplicatum Bory) in therapy group (c), there were improvements in the mucosal jejunum, the villi become length and compact.

3.4 Results of ZO-1 and Occludin Protein Expression of Rat Jejunum

Decreasing of ZO-1 and occludin protein expression of rat jejunum was indicator of tissue damage. This expression showed in brown color in jejunum tissue. It caused by production of ROS that can cause inflammation.

Figs. 8A and 8B respectively showed ZO-1 and occludin protein expression of rat jejunum. Both of ZO-1 and occludin protein in control rat were expressed. It showed a lot of brown color at preparate (a). Expressions of ZO-1 and occludin protein were decreased in IBD rats. The brown color was disappeared in preparate (b). After therapy with ethanol and ethyl acetate extract from brown seaweed (S. duplicatum Bory), both of ZO-1 and occludin protein were expressed again, showed in brown color (c).

Fig. 6  Average ratio of MDA level of jejunum.

Table 3  Profile of malondialdehyde (MDA) level of rat jejunum.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average MDA level (μg/mL)</th>
<th>Increasing MDA level to the control (％)</th>
<th>Decreasing MDA level to IBD rat (％)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.839 ± 0.067</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IBD</td>
<td>4.596 ± 0.429</td>
<td>149.92</td>
<td>0</td>
</tr>
<tr>
<td>Therapy</td>
<td>2.105 ± 0.035</td>
<td>14.46</td>
<td>54.20</td>
</tr>
</tbody>
</table>

Fig. 7  Histological picture of rat jejunum using HE staining with 100 × magnification.
a: Control rat; b: IBD rat; c: Therapy rat. Solid black arrows: villi. Dot black arrows: space.
The Potency of *Sargassum duplicatum* Bory Extract on Inflammatory Bowel Disease Therapy in *Rattus norvegicus*

Fig. 8  Expression of ZO-1 (A) and occludin (B) protein of tight junction rat jejunum with immunohistochemistry method using 100 × magnification.
a: Control rat; b: IBD rat; c: Therapy rat. Dot black arrow indicate ZO-1 expression. Solid black arrow indicate occludin expression.

4. Discussions

4.1 Profile of Malondialdehyde (MDA) Level of Jejunum from Control, IBD and Therapy Rats

MDA is the end product of membrane lipid peroxidation by ROS, so that measurements of MDA levels indirectly reflect level of free radicals. ROS is formed because indomethacin can weaken small intestine mucosal defense system indirectly so that it can cause infection. This infection can cause the activation of neutrophils and macrophages that produce ROS. In addition, ROS also caused by oxidation of the oxidation of metabolites indomethacin DMBI becomes iminoquinon and will indirectly stimulate the activation of macrophages and neutrophils into the tissue so that also induce ROS [9, 18].

MDA level of IBD rat that induced by indomethacin increased until 149.92% to the control rat (Fig. 6 and Table 3) because of indomethacin indirectly a major driver of ROS production in mucosal cells that will eventually happen lipid peroxidation.

Fig. 9 showed the reaction of MDA formation through lipid peroxidation process. Double bonds (C=C double bonds) of PUFA were a major target of hydroxyl radicals. The existence of double bonds (C=C) weaken the bond between carbon and hydrogen that facilitate the hydroxyl radical (OH•) in abstracting hydrogen atoms from PUFA. The initial phase of lipid (PUFA) peroxidation (RH) was hydrogen atom abstraction by hydroxyl radicals to form lipid radical (R•). Lipid radicals were highly unstable molecules that can react with O₂ to form lipid peroxyl radicals (ROO•). Lipid peroxyl radicals can react with PUFA to another, taking one electron and produces lipid hydroperoxide (hydroperoxides ROOH) and another lipid radical. Lipid peroxyl radicals can also undergo cyclization reaction producing cyclic peroxide which is adjacent to the carbon radical center. These radical generating molecules have a structure analogous to endoperoxide. Endoperoxide will then form the malondialdehyde [18, 19].

MDA levels of therapy rat (Fig. 6 and Table 3) decreased until 54.20% after treatment with ethanol and ethyl acetate extract from brown seaweed (*Sargassum duplicatum* Bory) because the content of compounds contained in the extract. They are flavonoids and phlorotanin as antioxidants. It was based on the structure of flavonoids and phlorotanin that have more than one phenol compound (having aromatic system and OH group) and have a conjugated double

Fig. 9  Formation reaction of malondialdehyde (MDA) through lipid (PUFA) peroxidation.
bond, where its structure was needed in scavenging free radicals. The existence of content in extract as antioxidants can scavenge of free radicals and prevent the formation of free radicals so that the process of lipid peroxidation can be suppressed. Scavenging reaction of free radicals by flavonoids and phlorotanin was shown in Figs. 10 and 11.

Mechanism of free radical scavenging by flavonoids and phlorotanin explained through Figs. 10 and 11. Based on the figures, there was an abstraction of hydrogen atom by free radicals (R•) produce phenoxy flavonoids (FlO•) and phenoxy phlorotanin (FrO•) radicals which have very low reactivity. This radical can be attacked again by free radicals to form phenoxy flavonoids and phenoxy phlorotanin radicals second. Because the phenoxy flavonoid and phenoxy phlorotanin radicals have conjugated double bonds, then occurred the delocalization of electrons to having stabilized structure and the free radicals were neutralized.

4.2 Histological of Jejunum from Control, IBD, and Therapy Rats

The villi structure of control rats were compact (mucosa in good condition) (Fig. 7a), Fig. 7b showed that mucosal damage in IBD rats were more severe. The condition of the villi IBD rats was abnormally, caused by ROS activity as free radicals. The villi react with non-radical molecules, thereby increasing the amount of ROS and lowering enzymatic antioxidant activity in cells that stimulate lipid (PUFA) peroxidation to increase the degree of damage.

Iminoquinon is one of the reactive metabolites of indomethacin which bind covalently with cellular nucleophiles such as GSH. GSH is an endogenous antioxidant. In this condition, GSH decrease, while the free radical (iminoquinon) was increase. This iminoquinon bind to the protein as a nucleophile (-SH or NH2) to form alkylated protein on the jejunum tissue. Producing of alkylated proteins cause hypersensitivity with type III hypersensitivity reactions [7, 20].

Ethanol and ethyl acetate extract of brown seaweed (S. duplicatum Bory) repair jejunum mucosal damaged (Fig. 7c). The content of flavonoids and antioxidants on phlorotanin brown seaweed act as a scavenger of free radicals that suppress the formation of ROS, perform scavenging reaction of free radicals and jejunum tissue can be repaired.

4.3 ZO-1 and Occludin Protein Expression of Jejunum from Control, IBD and Therapy Rats

Decreasing the protein expression of ZO-1 and occludin indicate tissue damage of the jejunum from IBD rats (Figs. 8A (b) and 8B (b)). It is caused by ROS that produce oxidized protein and protein fragmented [21]. The oxidized ZO-1 and occludin change the structure of jejunum protein. Damaging of this protein
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was also caused by hydrolysis reaction by some proteases. So that, tight junction of small intestine jejunum wasn’t compact. If compared with control rat (Figs. 8A (a) and 8B (a)), it showed that a lot of expressions of ZO-1 and occludin in preparete (brown color). It was indicated the tight junction still compact. Ethanol and ethyl acetate extract of brown seaweed (*S. duplicatum* Bory) repair ZO-1 and occludin of IBD jejunum rats that confirmed by immunohistochemistry technique (Figs. 8A (c) and 8B (c)). The expression of ZO-1 and occludin showed by brown colour in jejunum tissue. This result means that the ethanol and ethyl acetate extract of brown seaweed (*S. duplicatum* Bory) contained flavonoids and phlorotanin that act as antioxidants, followed by lowering ROS production, prevent the enzymatic hydrolysis reaction and also inhibit oxidized ZO-1 and occludin. The decreasing of ROS by flavonoids and phlorotanin reduce the damage of ZO-1 and occludin. This process involves the mechanisms of migration, proliferation, cell differentiation, and matrix synthesis extracellular. Growth factor (TGF-β, PDGF, VEGF and EGF) and its receptor would play a role for regenerating some epithelial cells [10].

5. Conclusions

MDA level of jejunum of white rats exposed to indomethacin after treated with ethanol and ethyl acetate extract from brown seaweed (*S. duplicatum* Bory) were decreased by 54.20%.

There were improvements of jejunum of white rats exposed to indomethacin after treatment with ethanol and ethyl acetate extract from brown seaweed (*S. duplicatum* Bory).

There were retrieval of ZO-1 and occludin protein in the jejunum of white rats exposed to indomethacin after treatment with ethanol and ethyl acetate extract from brown seaweed (*S. duplicatum* Bory).

Acknowledgments

The authors would like to thank Oganic Chemistry researcher team in Faculty of Sciences for phytochemistry analysis.

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Characteristics of Thyroid Hormones in Hypertensive Hispanic Population

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Abstract: The aim was to characterize thyroid function in Hispanic hypertensive population. Retrospective study of 1,023 individuals was performed, in which 68.23% had hypertension (74.21% women) and 37.25% had thyroid pathology. Age, weight, blood pressure, biochemical measurements were recorded, mean age: 53.02 ± 14.80 yr, mean weight: 83.05 ± 16.95 kg, thyrotropin-stimulating hormone (TSH) hypertensive patients: 5.55 ± 1.63 µIU/mL and without hypertension: 3.72 ± 1.60 µIU/mL. TSH range concentration distributed by sex indicates: 1.50-2.49 µIU/mL men, 2.50-5.01 µIU/mL women. TSH categories vs. age were positively associated ($r = +0.114, P = 0.044$) and hypertension by age was also correlated ($r = +0.178, P = 0.0001$). Significant positive association we found between TSH and diastolic blood pressure ($r = +0.197, P = 0.008$). Systolic and diastolic blood pressure vs. age were positively increasing ($r = +0.197, P = 0.008$). Prevalence of thyroid pathology in hypertensive subjects are hyperthyroidism 5.87%; hypothyroidism 20.34% (5.73% subclinic) and autoimmune disease 6.25%. High percentage of hypertensive population has concomitant thyroid diseases, more common in women (50-70 years). Systematic surveillance for occult thyroid dysfunction in patients with hypertension could prevent future cardiovascular disease.

Key words: Thyroid hormones, blood pressure, thyroid pathologies, patients, Hispanic.

1. Introduction

Extensive evidence indicates that the minimal and persistent changes in circulating thyroid hormone levels have a profound effect on cardiovascular system [1]. The existence of a relationship between hypertension and thyroid dysfunction has long been reported [2], yet the pathogeneses relationship between these conditions is less clear. Also, the relationship between traditional hypothyroid symptoms and biochemical thyroid function has not been fully delineated [3].

Thyroid dysfunction is relevant in some diseases as hypertension (HTA), atherosclerotic and lipid metabolism [2, 4-9]. Dysfunctions of the thyroid gland usually involve not enough production of thyroid hormone (hypothyroidism) or overproduction (hyperthyroidism) [2, 10-12]. Clinical and subclinical disease can alter the cardiac function and increment cardiovascular morbimortality, neither the heart nor the blood vessels function normally [13-15]. Disorders of the thyroid gland can worsen old cardiac symptoms or cause new ones, and can accelerate the underlying heart problem [16, 17].

In hypothyroidism, patients have been found a high incidence of hypertension, which was accompanied with increased peripheral vascular resistance [18]. The reported prevalence of hypertension in hypothyroidism varies between 0% and 50% [19].

The aim of this study was to characterize the frequency of thyroid dysfunction in Hispanic hypertensive patients and to evaluate the thyroid
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hormones levels related to the changes of blood pressure.

2. Materials and Methods

There were 1,023 individuals at a single cardiologic institution of San Luis (Argentine), who were performed between 2006 and 2008. The studied individuals included in the analysis had 20 years of age or older, and the weight was also recorded. The most of patients had hypertension and different thyroid pathology. The subjects who did not have a previous history of treatment thyroid disease were selected.

After time rest, the arterial pressure from each individual was measured using a standard mercury sphygmomanometer at upper right and left arm, then the mean value was calculated.

The VII Join National Committee (JNC-7) defined the hypertension as systolic blood pressure (SBP) higher than 140 mmHg and/or diastolic blood pressure (DBP) higher than 90 mmHg.

Thyroid gland dysfunction was determined by clinical examination and biochemical measurements of thyrotropin-stimulating hormone (TSH), levothyroxine (T4), free levothyroxine (fT4) and triiodothyronine (T3). Immune system dysfunction was determined by serologic measurements of antibodies to thyroglobulin and thyroid peroxidase. The prevalence of thyroid disease has also been estimated from clinical history.

Serum TSH concentration was analyzed using IFMA-MEIA hTSH Ultra (sensitivity 0.01 µUI/ml and total analytical variation < 5%). The reference range for TSH was defined as 0.47-5.01 µIU/mL. T4 was analyzed using IFMA-FPIA (reference range: 4.5-12.0 µg/dL), fT4 and T3 by IFMA-MEIA (reference range: 0.71-1.85 ng/dL and 0.76-1.42 ng/mL, respectively). Serum anti-thyroid antibodies (antiTPO, antiTG) analysis was performed by radioimmuno analysis technique (TPOAb CT RIA 100 TPT/100, TPO/50), SERODIA-AMC and SERODIA-ATG (Microtiter Particle Agglutination Test Kits for Detection and Titration of Thyroid Autoantibodies) are two semiquantitative assays for the detection of antimicrosomal and antithyroglobulin antibodies, respectively. The presence of these antibodies is abnormal and an indication of thyroid autoimmune disease.

The data were analyzed using the Statistical Package for the Social Sciences (SPSS), Version 12.0 for Windows (SPSS Inc., Chicago, IL). Descriptive statistical, frequencies, linear regression, correlation coefficients, with corresponding 95% confidence intervals (CI), Chi-square test with Yates’ continuity correction or chi-square for linear association were used. Differences among groups were evaluated using One-way Analysis of Variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test (Statistical Package GraphPad, version 3.02, San Diego, CA, USA). Means ± standard deviation were calculated and a probability of less than 0.05 was assumed to be significant.

3. Results and Discussion

Retrospective record review of 1,023 Hispanic middle class individuals was seen, 75.46% women and 24.54% men. Age and weight frequency distributions of studied patients are represented by histogram (Figs. 1a and 1b).

The mean age was 53.02 ± 14.80 years and 83.05 ± 16.95 kg of the mean weight. The studied individuals included in the analysis 68.23% had hypertension (74.21% women, 25.79% men) and 37.25% had different thyroid pathology.

The current study suggests that high percentage of our population studied had hypertension and thyroid pathology which affects an approximately a half of our hypertensive Hispanic individuals with prevalent percentage of adult females. Women are disproportionally impacted by hypertension and concomitant thyroid disease. Although these disadvantages between women and men could explain for some biological basis, such as, postmenopausal and differences in sex-specific life expectancy [20].
TSH assay is essential to establish a diagnosis of thyroid dysfunction. A frequency distribution of all studied patients is used to represent this set of data by listing the TSH values with their frequencies, mean: 5.31 ± 0.62 µUI/ml, median 2.41 µUI/ml. The histogram is also used to represent the set of data by listing the TSH values with their frequencies in patients with and without hypertension (Figs. 2a and 2b).

The means of TSH concentration were 5.55 ± 1.63 µUI/mL in hypertensive patients (women: 5.84 µUI/mL, men: 3.85 µUI/mL) and 3.72 ± 1.60 µUI/mL in individuals without hypertension (women: 3.63 µUI/mL, men: 4.18 µUI/mL). We also represented the percentage of both sex distributed with increasing range of TSH (less than 0.46, 0.47-1.49, 1.50-2.49, 2.50-5.01, > 5.01 µUI/mL and higher) (Fig. 3). The highest percentage was in TSH range 1.50-2.49 µUI/mL for men and 2.50-5.01 µUI/mL for women.

We used a frequency distribution of all studied patients to represent TSH values and their profiles vary between patients with and without hypertension. The mean of TSH concentration was in hypertensive women higher than men, above of reference range. Similarly, current reports estimate that it affects of the adult female population and a smaller percentage of adult males.
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The percentage of both sex distributed with increasing categories of TSH. Highest percentage of TSH range: 1.50-2.49 µUI/mL men and 2.50-5.01 µUI/mL women.

The association between TSH divided in five categories versus group of age indicates that increase of TSH is corresponding to higher age ($r = +0.114$, $P = 0.044$). The positive association between HTA and age is also correlated, and higher age is corresponding with increase level of HTA ($r = +0.178$, $P = 0.0001$).

The positive association between TSH, blood pressure and age indicates that increasing of TSH level and blood pressure are corresponding to higher age.

Mean systolic blood pressure and mean diastolic blood pressure by five categories of TSH in hypertensive patients are shown in Fig. 4.

Significant differences were observed between mean systolic and mean diastolic blood pressure by categories of TSH in hypertensive patients compared with no hypertensive patients (Figs. 5a and 5b).

![Fig. 3 The percentage of both sex distributed with increasing categories of TSH. Highest percentage of TSH range: 1.50-2.49 µUI/mL men and 2.50-5.01 µUI/mL women.](image)

![Fig. 4 Systolic and diastolic blood pressure versus five categories of TSH (0.01- > 5.01 µUI/mL) in hypertensive patients. The values are mean ± SD. • : SBP; ▲ : DBP.](image)

![Fig. 5 Systolic blood pressure and diastolic blood pressure by categories of TSH (0.01- > 5.01 µUI/mL) in hypertensive patients compared with no hypertensive patients. The values are means ± SD. The data show a statistically significant differences $P < 0.0001$ (One-way Analysis of Variance, ANOVA), followed by Tukey-Kramer Multiple Comparisons Test: *** $P > 0.001$, * $P > 0.05$. (a) SBP; (b) DBP. • HTA ▲ control.](image)
The mean systolic and diastolic blood pressure by categories of TSH in hypertensive individuals was significant higher than no hypertensive patients.

We investigated associations of thyroid function with systolic and diastolic blood pressure in hypertensive subjects. The main outcome measures were mean systolic and diastolic blood pressure for hypertension (> 140/90 mmHg) according to five categories of TSH. There was an increase in systolic and diastolic blood pressure with increasing concentration of TSH. Significant positive association we found between TSH and diastolic blood pressure ($r = +0.197, P = 0.008$) and no significant correlation between TSH level and systolic blood pressure.

This association was significant when only hypertensive subjects were considered. The clinical significance of our findings is not clear. However, we known that these associations are sufficiently important to influence future risk of cardiovascular disease and could be checked with systematic clinical studies.

Several authors have reported a positive association between TSH levels and blood pressure [2, 17, 21-24]. On the other hand, no such associations were founded in other studies [25-28], however thyroid dysfunction commonly were accompanied with other cardiovascular failure [29], atrial fibrillation in subclinical hypothyroidism [30], myocardial contractility [31, 32] and metabolics syndromes [28].

In other study, mean systolic and diastolic blood pressure did not differ between individuals with subclinical hypothyroidism and euthyroid subjects [33]. The high prevalence of hypertension with increasing concentrations of TSH suggests that could be due to a genetic susceptibility [34]. Therefore, other results could not support this hypothesis [35].

Thyroid hormone profile in patients with hypertension divided into five groups by categories of TSH was described. The biochemical characteristics of thyroid function were studied in the population, the data are shown in Table 1. Mean levels of T4 hormones decreased within the normal range while TSH hormone was increasing. A decrease in mean plasma T3 was observed. Mean plasma T3 was higher than reference values on lower TSH values (< 0.46 µIU/mL). There were no relevant changes in TSH, T4, T3 hormones levels between the groups.

The association of T3, T4 and fT4 with range of TSH concentration was also studied, there was an inverse association ($r = -0.252, P = 0.0001; r = -0.331, P = 0.0001; r = -0.387, P = 0.005$, respectively).

We stratified in Table 2 the thyroid function test of TSH, T4 and T3 in relation to patients’ age in years (less than 29 yr, 30-49 yr, 50-69 yr, 70 yr and higher). TSH levels vary with age, increased markedly in the age from 30 yr to 69 yr. Mean plasma TSH was higher than reference values. The rise of TSH with age decreased within reference range when persons had above 70 yr of age. Mean levels of T4 and T3 hormones were within the normal range. There were no significant changes in T4 and T3 hormones levels between the groups.

The prevalence of thyroid dysfunction of subjects over 30 yr old and especially between 50 yr and 69 yr the thyroid pathology was markedly higher than other groups’ age.

Mean systolic and diastolic blood pressure for four categories of age were calculated, there was an increase in systolic and diastolic blood pressure with increasing age ($r = +0.410, P = 0.0001; r = +0.285, P = 0.0001$, respectively).

<table>
<thead>
<tr>
<th>Age</th>
<th>TSH (µIU/mL)</th>
<th>T4 (µg/dL)</th>
<th>T3 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01-0.46</td>
<td>11.31 ± 1.22</td>
<td>2.08 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>0.47-1.49</td>
<td>8.02 ± 0.37</td>
<td>1.14 ± 0.05</td>
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</tr>
<tr>
<td>1.5-2.49</td>
<td>7.89 ± 0.40</td>
<td>1.11 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>2.50-5.00</td>
<td>7.04 ± 0.25</td>
<td>1.15 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>&gt; 5.01</td>
<td>6.10 ± 0.36</td>
<td>0.97 ± 0.04</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>TSH (µIU/mL)</th>
<th>T4 (µg/dL)</th>
<th>T3 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;29</td>
<td>2.57 ± 0.62</td>
<td>5.81 ± 1.22</td>
</tr>
<tr>
<td>30-49</td>
<td>5.63 ± 2.40</td>
<td>7.43 ± 0.37</td>
</tr>
<tr>
<td>50-69</td>
<td>5.80 ± 1.14</td>
<td>7.58 ± 0.27</td>
</tr>
<tr>
<td>&gt;70</td>
<td>3.90 ± 0.86</td>
<td>7.29 ± 0.70</td>
</tr>
</tbody>
</table>
Thyroid diseases are quite common in patients with hypertension and often remain undetected. TSH level stratified by age was higher to 30-69 yr group; similarly the prevalence of thyroid dysfunction was increased in this range aged. Thyroid dysfunction is known to be more common in women than in men, and women generally use the health care system more than men, especially in the age group of 50-69 yr. The importance of the recognition of the effects of thyroid disease on the heart also derives from the observation that restoration of normal thyroid function most often reverses the abnormal cardiovascular hemodynamics.

The present study has also been conducted to determine the distribution of different thyroid pathology in the community studies (Table 3). The prevalence of elevated TSH levels (normal range: 0.47-5.01 µUI/mL) in this population was 21.80% (19.23% women, 2.57% men), and the prevalence of decreased TSH levels was 8.20% (7.69% women, 1.23% men). The prevalence in hypertensive patients’ overt hyperthyroidism was 5.87% (5.01% women, 0.86% men). The prevalence of overt hypothyroidism was 20.34% (14.61% clinic, 5.73% subclinic), 17.91% women compared with less than 2.43% men. The presence of thyroid antibodies was determined in 153 patients (14.95%) and was evident (greater than 1:100) in 64 hypertensive patients (6.25%), the autoimmune mechanism is commonly associated with other forms of thyroid disease. The goitre type was diagnostic and classified as diffuse in seven patients (0.68%), multinodular in 15 (1.47%) and solitary nodule in three women (0.29%). Ten subjects (0.98%) had clinical features suggestive of malignancy (nine women).

In patients with overt hypothyroidism, the lack of T₄ feedback leads to TSH levels > 5.01 µUI/mL, whereas in milder or subclinical hypothyroidism (5.73%), the TSH levels are between 2.5 and 5.01 µUI/mL, with normal T₄ and T₃ levels. We detected important degrees of hypothyroidism on the basis of elevated serum TSH levels. The prevalence of overt hypothyroidism was high percentage, rising in women and less in men. TSH levels vary with age in both sexes and shown an increased markedly in women above 30 years old. In contrast, all forms of hyperthyroidism are accompanied by TSH levels that are suppressed to < 0.47 µUI/mL. The prevalence of overt hyperthyroidism was less than hypothyroidism in hypertensive individuals, rising to women compared with men. Thus the TSH assay is the appropriate initial test to screen for thyroid dysfunction in a variety of clinical situations known to be affected by thyroid disease, as well as to confirm a suspected diagnosis and follow the response to treatment. Various authors have suggested that the reference range for TSH could be narrowed.

Evidence suggests that hyperthyroidism and hypothyroidism produce opposite cardiovascular consequences, however the vascular morbi-mortality occurs in both overt and subclinical thyroid diseases [36]. In almost all cases these cardiovascular changes are reversible when the underlying thyroid disorder is recognized and treated.

TSH levels above 5.01µUI/mL were shown to reflect
a significant lowering of circulating T₄ levels, and showed a strong association with thyroid antibodies in both sex. The prevalence of thyroid antibodies found indicating that the autoimmune mechanism is one of the most common forms of thyroid disease. Thyroid cytoplasm antibodies present in hypertensive individuals did not vary significantly with age and increased markedly in women over 30 years of age. Goitres were more common in women than in men, and most found in younger rather than in older women.

4. Conclusion

Our study has demonstrated that a higher percentage of the population studied with hypertension have concomitant thyroid disease.

The prevalence of abnormal biochemical thyroid function reported here is substantial and confirms previous reports in others populations. Individual symptoms are not very sensitive, but patients who report multiple thyroid symptoms warrant serum thyroid testing. These results confirm that thyroid dysfunction is common, may often go undetected, and may be associated with adverse health outcomes that can be avoided by serum TSH measurement.

Abnormal circulating thyroid hormones levels modify the normal function of cardiovascular system, these effects including changes on cardiac systolic and diastolic function and peripheral vascular resistance.

Thyroid dysfunction is more common in women than in men, and women generally use the health care system more than men. The percentage of hypothyroidism in our patients with hypertension is high.

Our data also show a positive correlation of diastolic blood pressure across the range of TSH, and increasing prevalence of hypertension with increasing TSH level, which could be implications for cardiovascular health.

In conclusion, our results in conjunction with previous observations demonstrate that the alteration in thyroid hormones might affect the complex physiological processes that regulate the cardiovascular system. In addition, new studies are necessary to elucidate the mechanisms which thyroid gland is related with regulation and/or modulation of blood pressure.

The effect of these two conditions could be linked by a common immunogenetic susceptibility, variations of gene expression has also been proposed, and a number of studies have indicated that the elucidation of this association could advance the understanding of the pathophysiology and treatment of hypertension. Systematic surveillance for occult thyroid dysfunction in patients with hypertension could prevent the hemodynamic exacerbation of cardiovascular failure.

References

Characteristics of Thyroid Hormones in Hypertensive Hispanic Population


Condition Metabolic Balance Disturbance in Patients with Cor Pulmonale: Prevalence, Diagnosis, Risk Factors

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Abstract: To study the risk factors for the development of cor pulmonale (CP) in patients with chronic pulmonary diseases in the Republic of Uzbekistan, the disbalance in parameters of stable metabolites oxides of the nitrogen (SMNO), of blood oxygen saturation (SaO₂), of respiratory function and on lipid peroxidation of cell membrane were measured. The authors evaluated the effect of base therapy and ozone therapy (OT) in patients with CP. Basis therapy and OT resulted in good parameters of SMNO, SaO₂ and metabolic activenss of cell, significant positiveness of hypoxemia and parameters of pulmonary ventilation ability and hemodynamics of pulmonary arteria.

Key words: Pulmonary arterial hypertension, ventilation lung capacity, oxidative stress, oxides of the nitrogen, ozone therapy.

1. Introduction

The growing interest has been observed among the pulmonological specialists for the problem of assessment of the right and left heart ventricle functions for last decades [1, 2]. Caused by alveolar hypoxia the pulmonary arterial hypertension (PAH) is finished by formation of cor pulmonale (CP) in chronic pulmonary diseases [3].

Now, it is reliably established that ventilation-perfusion disorders, alveolar hypoxia and hypoxemia lead to considerable changes in lipid peroxidation (LP) in the tissue membrane structures [4]. It is connected that, first of all, with local hypoxia, changes of intermolecular links result in decrease of mechanical stability and erythrocyte’s life time [5, 6].

The modern concept of chronic obstructive pulmonary diseases (COPD) developed by the WHO experts, is based that this disease is related to those diseases, whose development can be prevented (primary prevention) and be treated rather successfully (secondary prevention); the clinical course severity and prognosis are often defined by the extrapulmonary manifestations of disease [7, 8].

The search of alternative nonmedical methods of treatment for CP is important too. Ozonatherapy is one of the methods of therapy for patients with COPD complicated with cor pulmonale. According to the data of Chazov [9, 10] in the lungs after inspiration of ozone or air with high oxygen concentration the activity of antioxidant (AO) system increases considerably. Purpose is to study the risk factors of the cor pulmonale development at the patients with chronic pulmonary diseases in Uzbekistan; effect of different regimens of therapy on the parameters of ventilation-perfusion pulmonary functions, of stable metabolites’ oxides of the nitrogen and oxidative activity of cellular membranes at the patients with COPD complicated with CP.

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2. Methods

Pulmonological screening included interview under the questionnaire of Pauwels et al. [11]; doppler echocardiography with use of ultrasound device Shimadzu 500A and Toshiba SSH 60A (Japan) according to the recommendations of the American Society of echocardiographers by Khatle and Angelson (1985): on the basis of findings of doppler echocardiography the pulmonary hypertension without right ventricle dilatation was assessed (RVD, level of average pulmonary arterial pressure—PAPav was more than 25 mm Hg) and with RVD (thickness of RV posterior wall was less than 5 mm, at anterior-posterior size of RV more than 2.5 cm); the obligatory parameters of expiration peak speed were measured in relation to sex, age and height.

During treatment there were 54 patients studied with COPD, complicated by CP, and 30 healthy persons (HP) as volunteers.

The patients with COPD complicated by pulmonary arterial hypertension (groups 1a and 1b) and patients with COPD complicated by CP with RVD (groups 2a and 2b) were studied. Depending on the selection of therapy, all patients were divided into the following groups: 1a group (16 patients) and 2a group (14 patients) received basic therapy (BT) on GOLD (2006); 1b group (15 patients) and 2b group (11 patients) additionally to BT received ozonetherapy (OT).

The examinations were performed at the day of admission and on the 10 days, after ten-day performance of the different schemes of treatment with use of standard mean doses of BT and OT in form of intravenous administration of ozone-acid mix in a physiological solution (1,000 mg/L).

The erythrocyte membranes were investigated with use of reaction of lipid free oxidation, by parameters of a primary product of lipid peroxidation (LP), which is intermediate products of peroxide chemiluminescence (Chl, imp/s/mg), malon dialdehyde (MDA, nmol/mg) and also by activity of the components of physiological antioxidant system including antioxidant enzymes: superoxidismutase (SOD, un/min/mg), catalase (CAT, mmol/H2O2/min/mg). The level of oxides of the nitrogen (NO) metabolites was measured on the basis evaluation of stable metabolites’ NO summary concentration nitrates and nitrite (SMNO) in the deproteinized plasma with using the spectrtrophotometric method, blood oxygen saturation (SaO2) was measured by pulsoximetry method.

Pulmonary ventilating ability (PVA) was studied on the device Medikor (Hungary), with the evaluation of the forced expiration volume per 1 sec (FEV1, %), forced vital capacity (FVC, %) and Tiffno index (FEV1/FVC, %). The results are processed with use of the software package Excel, there was calculated average arithmetic value and standard error (M ± SD). Reliability of differences obtained in the compared groups, was estimated with use of student’s t-criterion. The differences between studied parameters were considered to be statistically reliable at $P < 0.05$.

3. Results

At the patients with COPD complicated by CP before treatment there was found lower of the parameter of blood oxygen saturation in all the groups in comparison with similar parameters in healthy volunteers by 11.9, 13.3, 14.6 and 15.2% ($P < 0.001$).

Before treatment in the patients with COPD complicated by CP there was noted reduction of SMNO in blood plasma and blood oxygen saturation during development of PH and RVD (in comparison with parameters of healthy people, $P < 0.05$) (Fig. 1).

The level of average pulmonary arterial pressure before treatment was raised by 50.5, 49.01, 59.9 and 57.7% in comparison with similar parameters of healthy people ($P < 0.005$). The application only of BT had no significant influence on the investigated parameters and after the treatment in 1a and 2a groups, the received data in comparison with similar parameters before the treatment were doubtful.

The results of clinical examinations of the patients with COPD complicated by CP showed (Table 1) ventilation-perfusion disorders, intensification of LP
processes which were response to hypoxia and during cellular adaptation expressed by marked severe clinical course of disease.

During the complex treatment on the background of BT with ozonetherapy at the patients with COPD complicated by CP with pulmonary arterial hypertension and RVD in 1b and 2b groups, the parameters of pulmonary ventilaton ability have been considerably increased in 1b and 2b groups, respectively: FEV1 by 9.1-7.5%, FVC by 8.4-7.2%, FEV1/FVC by 8.7-7.4%, PAPav by 15.7-13.1% (in comparison with parameters before treatment, \( P < 0.05 \)).

After treatment there was observed MDA reduction by 16.5-15.9%; CHL by 17.7-16.1%, as well as increasing SOD by 17.1-16.3%, CAT by 14.5-13.6% \( (P < 0.05, \text{reliability of differences with similar parameters before treatment}) \).

**4. Discussion**

Lower of the parameters of blood oxygen saturation and SM\(_{NO}\) induce growing intensification of the processes changes in lipid peroxidation in the tissue membrane structures and vascular remodeling [6]. Dennis and other authors showed that hypoxemia is a factor of the pathogenetic mechanism of vascular wall damage and endothelial dysfunction. The powerful endogenous NO vasodilator inducing resolution of the vascular wall smooth muscles participates actively in regulation of the systemic and pulmonary vascular resistance.

![Fig. 1](image)

**Fig. 1** Dynamics of the parameters pulmonary ventilation-perfusion ability and SM\(_{NO}\) in patients with COPD complicated by CP during complex treatment.

**Table 1** Change of the activity of some enzymes of the system LP/AO in the erythrocyte membranes during complex treatment at the patients with COPD complicated by CP (M ± m).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy persons (n = 30)</th>
<th>Basic therapy</th>
<th>BT + OT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA, Nmol/mg/protein</td>
<td>0.310 ± 0.01</td>
<td>0.495 ± 0.03**</td>
<td>0.531 ± 0.02**</td>
</tr>
<tr>
<td>Chl, imp/s/mg</td>
<td>48.1 ± 3.5</td>
<td>66.5 ± 5.7**</td>
<td>63.9 ± 4.7**</td>
</tr>
<tr>
<td>SOD, un/min</td>
<td>225.7 ± 4.3</td>
<td>127.4 ± 3.6**</td>
<td>114.5 ± 4.0**</td>
</tr>
<tr>
<td>CAT, mcmol/min/mg</td>
<td>410.8 ± 6.8</td>
<td>218.7 ± 10.8**</td>
<td>204.2 ± 4.8**</td>
</tr>
<tr>
<td>PAPav, mmHg</td>
<td>14.0 ± 2.0</td>
<td>28.2 ± 0.6**</td>
<td>28.1 ± 0.7**</td>
</tr>
</tbody>
</table>

Numerator: parameters before treatment (reliability of the differences with parameters of healthy persons); denominator: parameters after treatment (reliability of differences with parameters before treatment); **: \( P < 0.005 \); *: \( P < 0.05 \).
Unsaturated fat acids are substrates for free radicals in the hydrophobic part of membrane, and the accumulation of free radicals results in disorders in the membrane structures [11, 12]. In our researches increase in LP activation before treatment was not compensated by increase in parameters of antioxidant system.

Before treatment the established reduced parameters of pulmonary ventilation ability, blood oxygen saturation and enzymatic activity of antioxidant system induced intensity of the processes of cell membrane remodeling, endothelial vessels and myocardium, which coincided with the statements of Hansell [13], and Tsoyi et al. [14]. The low parameters of blood oxygen saturation are one of the main reasons of pulmonary vasoconstriction and pulmonary arterial hypertension.

Thus, the data received during the treatment indicate that after the complex therapy performed there was noted decrease in oxidative stress, positive shifts in the parameters PVA, SaO₂, SMNO and PAPav.

The inclusion of basic therapy COPD in the complex with ozonotherapy at the patients with COPD complicated by CP with pulmonary arterial hypertension and RVD provides not only powerful vasodilatation effect on the vessels of the small circle of circulation, but also reduces hypoxemia and CP progressing.

5. Conclusions

Oxidative damages in the erythrocyte membranes due to tissue hypoxia at the patients with COPD complicated by CP, as complex body adaptation responses, have not only local, but also system character, about what the damages in the system oxidant/antioxidant with shift to the oxidants in the peripheral blood indicated.

At the patients with chronic obstructive pulmonary disease complicated by CP during the performance of complex treatment with ozonotherapy additionally to the basic therapy for 10 days there were observed reduction of the oxidative stress, parameters SMNO, hypoxemia and parallel improvement of the parameters of pulmonary ventilation ability and hemodynamics of pulmonary arteria ($P < 0.05$).

References


Effect of Heat Treatment Combined with Shoot Tip Culture on the Virus-Free of Arena Strawberry

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Abstract: With Arena strawberry as experimental materials, heat treatments combined with shoot tip culture were used to explore their detoxification effects. The results indicated that if not heated, splitting shoot tips directly could not completely remove viruses. On the other hand, it will be obviously improved after shoot tips are heated. Incubators heat treatment (inconstant temperature) with shoot tip culture, constant temperature water bath incubation process with meristem-tip culture, hot air treatment (constant temperature) with apical meristem culture were three methods tested in searching for virus-free of Arena strawberry in this experiment. And their virus-free rates were 78.9%, 86.0% and 50.0% respectively. Of the three methods, the second, constant temperature water bath process with shoot tip culture, with 78.7% survival, is the best. Thus, it can be inferred that constant temperature water bath treatment combined with shoot tip culture might be the most appropriate method to remove viruses of Arena strawberry.

Key words: Arena strawberry, heat treatment, shoot tip, virus-free.

1. Introduction

Strawberry (Fragaria ananassa Duch.) is one of perennial evergreen herbaceous plants. As a rose family plant, it belongs to strawberry genus. Strawberry is a traditional and famous fruit in China, and its cultivation area and yield are only lower than grapes’ in production of berry fruits worldwide [1]. Domestic and overseas consumers give special preferences (favors) to strawberry widely because of its excellent characteristics, such as delicious flavor, rich in variety of nutrients and vitamins and so on [2]. However, strawberry’s cultivation and reproduction always take the way of vegetative propagation, such as making use of creeping stems or separable plants. Therefore, the infection rate of multiple virus diseases is rising continuously year by year. Viruses do harm to vegetative reproduction of strawberry more and more seriously, with plant dwarfed, runners reduced and fruit quality tended to be inferior, cutting up to 30%-80% in production, even causing plant death [3].

In China, due to the strawberry viral diseases, the yield of strawberry cut by 350 thousand tons each year. The direct economic loss reached RMB 3 billion [4]. Strawberry plants have no immune abilities to virus themselves, and the damage of virus has the characteristics of systematic infection. At present, no special drug can cure strawberry plants’ virus diseases except for using virus-free seedlings to breed strawberry plants [5]. Therefore, it is significantly important for the development of strawberry industry in China to strengthen the researches on strawberry virus-free technology, and to breed and popularize virus-free strawberry plants.

At present, there are three main approaches to removing strawberry viruses, namely, heat-treatment, anthers culture and meristem culture [6-11]. The most widely used and effective approach to getting virus-free strawberry is shoot tip tissue culture all
around the world. We know virus-free (detoxification) rate can reach 100% by cutting off shoot tip 0.2-0.3 mm in independent meristem culture, but this puts forward to a higher technical requirement which could not be mastered easily. Meanwhile, it is time-consuming. All mentioned above result in serious waste of manpower and material resources [12-15]. What is more, in the traditional heat treatment method, strawberry plants need to be put in an environment of high temperature and humidity for a long time. The plants’ vigor is inclined to reduce. Some plants even tend to die. In addition, if the materials through heat treatment are used in tissue culture, it will be more easily lead to pollution for the disinfection of surface is not thorough [16, 17]. Most scholars have come to an agreement that virus-free rate could reach 100% through anther tissue culture in the earlier researches. However, in the course of anther tissue culture, the in vitro cultures influenced by internal and external factors often come into being variants and make the original characteristics changed, including the variations of chromosome structures, chromosome numbers and on DNA molecular level [18-20].

There have been many reports on the research of the virus-free method of heat treatment combined with shoot tip culture, which have many improvements and innovations [21-23]. But it is rarely reported that these ways applying on Arena strawberry. Arena strawberry, introduced from Spain, is a cultivar of high quality and yield, has long shelf life in storage and transportation. And it can fructify during all seasons [24]. Different methods of heat treatment combined with shoot tip tissue culture were studied here, and effects of different treatment combinations on virus elimination of Arena strawberry were also discussed. The virus-free technical route for Arena strawberry had been established, which provided reliable references for Arena strawberry virus-free production in practical application and could be of great value in reducing accumulation of virus diseases in the process of vegetative propagation.

2. Materials and Methods

2.1 Materials

Arena strawberries required in this experiment were planted in the plastic canopy of Sichuan Agricultural University Teaching Area. On June 20, 2010, young plantlets through newborn runners of Arena strawberry were cut off and transferred into flower pots and cultivated for two to three months until they grew strong enough to be used.

2.2 Methods

2.2.1 Initial Virus Detections

In this experiment, electron microscope method [25, 26] was used to detect viruses of Arena strawberry. And samples were delivered into electron microscope measure room of Sichuan Agricultural University for testing. The main method was negative dyeing: leaves treated with special dye were observed respectively in 15,000 ×, 20,000 ×, and 30,000 × scopes, where virus particles could be clearly seen in the nucleus and the cytoplasm if plants were infected.

2.2.2 Heat-Treatment Methods

2.2.2.1 Hot Air Treatment under Inconstant Temperature

Potted plants (Arena strawberry) were put into the artificial climate box (RXZ-430, made in Ningbo of China), 38 °C, 16 hours during the day, 25 °C, eight hours during the night, air humidity maintained at 50%-70%. The soil humidity in the pots sustained the strawberry seedlings’ growth well enough, neither to be withering, nor to be too wet, for two weeks.

2.2.2.2 Water Bath Treatment under Constant Temperature

Chose strong creeping stems from Arena strawberry whose leaflets had not yet been fully unfolding, cut down the tip into 4-5 cm long, and get cleaned. Put it in 40 °C constant temperature water bath pot and treated for four hours.

2.2.2.3 Hot Air Treatment under Constant Temperature

Treated runners of Arena strawberry with 40°C
constant temperature in drying oven for 10 mins.

2.2.3 Stripping Shoot Tips and Tissue Culture

2.2.3.1 Stripping Shoot Tips

The treated creeping stems 3-4 cm in length were taken. Firstly, their surfaces’ dirty was cleaned and flossed with 1.0% washing powder water, then washed 2-4 hours with running water. They were taken into inoculating room and put on the super-clean worktable (SW-CJ-ZF made in Suzhou of China). Thenceforth, the samples were rinsed for 30 seconds with 75% alcohol, washed for 2-3 times with sterilized water, then processed with 0.1% of HgCl₂ for 2-3 min, with constantly shaking, and subsequently rinsed 4-5 times with sterilized water in order to remove HgCl₂. Finally, with the help of the anatomical lens (XTD-06 made in China), stripped off these lobules around the shoot tip from outside to inside until the growing point was revealed. About 0.3-0.5 mm in length of the shoot tip which contained 1-2 leaf primordia was cut down. The shoot tip was inoculated into the differentiation medium (starting medium) (Murashige & Skoog (MS) + 0.1 mg·L⁻¹ Gibberellins Aid (GA₃) + 0.2 mg·L⁻¹ Indolent Butyric Acid (IBA) + 1.0 mg·L⁻¹ 6-Benzylaminopurine (6-BA) (pH 5.8)) [27, 28], cultivated in 25 ± 1°C, 1,500-2,000 l ×, about 12-15 hours per day. Untreated shoot tips were used as controls.

2.2.3.2 Successive Transfer Culture

When the growing point developed into a bud group (formation of adventitious bud) about 1 cm after 1-2 months, which contained 4-5 buds, was transferred into subculture medium (MS + 0.5 mg·L⁻¹ 6-BA) [29, 30]. New medium was replaced every three weeks.

2.2.3.3 Rooting Culture

New buds were transferred 3-4 times before they could develop into tube seedlings about 2-3 cm that had no roots. They were transferred into rooting medium (MS + 0.2 mg·L⁻¹ Indolent Acetic Acid (IAA) [31, 32]) for root inducing.

2.2.4 Detection of Effects on Virus-Free

Young leaves were used to index the effects on virus-free of Arena strawberry, which were collected from seedlings that multiple mud clumps grew into through many divisions and transfers. Detection method was same as the primary virus detection.

2.3 Observations and Statistics

Shoot tips were inoculated in initial medium for three days before we began to watch and record the growth situation of shoot tip meristem. The pollution rate, survival rate, and virus-free rate of shoot tips were calculated accordingly.

\[
\text{Pollution rate} = \frac{\text{the number of pollution}}{\text{the number of inoculation}} \times 100\%
\]

\[
\text{Survival rate} = \frac{\text{the number of survivor}}{\text{the number of inoculation} - \text{the number of pollution}} \times 100\%
\]

\[
\text{Virus – free rate} = \frac{\text{the number of virus – free}}{\text{the number of survival}} \times 100\%
\]

3. Results and Analyses

3.1 Turning Green and Expansion of the Tip Meristem

Different heat therapies in combination with apical meristem culture of Arena strawberry all had great effects on starting and turning green of tip-meristem. Table 1 presents that most shoot tips started to turn green and finished turning green during the initial 10 days. The number of shoot tips starting to turn green in constant temperature water bath heat treatment or in control group was significantly more than in inconstant temperature hot air treatment or constant temperature hot air treatment. After 14 days, the number of shoot tips starting to turn green in constant temperature water bath heat treatment or control group was significantly more than others at a level of 0.05, while the number of shoot tips starting to turn green in constant temperature hot air treatment was significantly less than others at a level of 0.01. It was observed that those shoot tips which had not finished turning green finally would not survive over 14 days. Obviously, there was a positive correlation between the counts of shoot tips starting to turn green and the final growth rate. Besides, among the three treatment combinations (groups), the number of tip-meristem turning green eventually between the
Effect of Heat Treatment Combined with Shoot Tip Culture on the Virus-Free of Arena Strawberry

### Table 1  The effect on turning green and expansion of shoot tips under different treatments.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>The number of inoculation</th>
<th>The number of turning green (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3-6 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7-10 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-14 days</td>
</tr>
<tr>
<td>Group 1(^d)</td>
<td>30</td>
<td>5.0 AB b</td>
</tr>
<tr>
<td>Group 2(^e)</td>
<td>30</td>
<td>4.3 AB bc</td>
</tr>
<tr>
<td>Group 3(^f)</td>
<td>30</td>
<td>0.7 B c</td>
</tr>
<tr>
<td>Group 4(^g)</td>
<td>30</td>
<td>10.0 A a</td>
</tr>
</tbody>
</table>

\(\^d\) Group 1 means hot air treatment under inconstant temperature; \(\^e\) Group 2 means water bath treatment under constant temperature; \(\^f\) Group 3 means hot air treatment under constant temperature; \(\^g\) Group 4 means control group. \(\text{LSD difference significant inspection method, the same letter indicated no significant difference. A, B, C = 0.01; a, b, c = 0.05; data separated within column represent significant levels of A, B, C at 0.01 and a, b, c, at 0.05 by Duncan’s multiple range test. Above explanations are the same to the ones in Tables 2 and 3.}\)

### Table 2  Pollution rate, survival rate, and the number of multiple bud clumps differentiation in the process of shoot tip culture.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>The number of inoculation</th>
<th>The number of pollution (^b)</th>
<th>The rate of pollution (^b) (%)</th>
<th>The rate of survival (^b) (%)</th>
<th>The number of clustered shoots differentiation (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1(^d)</td>
<td>30</td>
<td>2.0 A b</td>
<td>6.7 A b</td>
<td>64.3 AB bc</td>
<td>10.3 AB b</td>
</tr>
<tr>
<td>Group 2(^e)</td>
<td>30</td>
<td>3.0 A ab</td>
<td>10.0 A ab</td>
<td>78.7 AB ab</td>
<td>10.2 B b</td>
</tr>
<tr>
<td>Group 3(^f)</td>
<td>30</td>
<td>4.0 A a</td>
<td>13.3 A a</td>
<td>53.3 B c</td>
<td>9.9 B b</td>
</tr>
<tr>
<td>Group 4(^g)</td>
<td>30</td>
<td>2.0 A b</td>
<td>6.7 A b</td>
<td>89.0 A a</td>
<td>10.8 A a</td>
</tr>
</tbody>
</table>

(\(^d\) Group 1 means hot air treatment under inconstant temperature; \(^e\) Group 2 means water bath treatment under constant temperature; \(^f\) Group 3 means hot air treatment under constant temperature; \(^g\) Group 4 means control group. \(\text{LSD difference significant inspection method, the same letter indicated no significant difference. A, B, C = 0.01; a, b, c = 0.05; data separated within column represent significant levels of A, B, C at 0.01 and a, b, c, at 0.05 by Duncan’s multiple range test. Above explanations are the same to the ones in Tables 2 and 3.}\)

constant temperature water bath heat treatment and the inconstant hot air treatment had no significant differences. But in the aspect of time, the former one was much shorter. Above all, except for the control group, constant temperature water bath heat treatment was the most appropriate for the induction of shoot tips’ turning green in this test. It would be helpful for shoot tip meristem expansion and came into being callus, which was the basis on production of bud groups and clustered shoots.

### 3.2 Effect of Different Heat Treatments on Arena Strawberry Shoot Tip Culture

Previous heat treatments had certain impact on the survival rate and the number of clustered shoots differentiation of shoot tip culture. From Table 2, it shows that all treatments showed extremely no significant difference in the aspect of pollution ratio. That was to say, heat treatment for Arena strawberry meristem culture had no significant influence on pollution. In the aspect of survival ratio (rate of survival), control group was significantly higher than others at the level of 0.01, survival ratio reached 89.0%. And constant temperature hot air treatment was the lowest, only reached 53.3%. While there was no obvious difference between inconstant temperature hot air treatment and constant temperature water bath heat treatment. The two combinations were superior (better) to (than) constant temperature hot air treatment. Perhaps the high temperature in constant temperature hot air treatment reduced the shoot tips of Arena strawberry to severe dehydration, consequently led to lower growth activity. The number of clustered shoots differentiation in control group was significantly higher than the number of others at the lever of 0.05. While at the lever of 0.01, three groups were not considerably different between each other, which indicated that heat treatment might make the number of clustered shoots differentiation in meristem culture decreased.

### 3.3 Detection (Appraisal) of Virus-Free Effect

From Table 3, we could conclude that the effect of constant temperature water bath heat treatment on the virus-free was the best, which was significantly higher than other three. There was no obvious difference.
Table 3  Effect of electron microscopy detection on virus-free.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>The number of survival</th>
<th>The number of virus-free</th>
<th>Virus-free percent b (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 d</td>
<td>19.3</td>
<td>15.0</td>
<td>78.9 AB b</td>
</tr>
<tr>
<td>Group 2 e</td>
<td>23.6</td>
<td>20.3</td>
<td>86.0 A a</td>
</tr>
<tr>
<td>Group 3 f</td>
<td>16.0</td>
<td>8.0</td>
<td>50.0 B c</td>
</tr>
<tr>
<td>Group 4 g</td>
<td>26.7</td>
<td>12.0</td>
<td>46.4 B bc</td>
</tr>
</tbody>
</table>

between constant temperature hot air treatment and control group, with virus-free ratio 50.0% and 46.4% respectively. This indicated that constant temperature hot air treatment had not considerably increased the efficiency of virus-free. This perhaps had something to do with temperature process and time setting. Speaking of virus-free effect, constant temperature water bath heat treatment could be the best processing among three treatments.

4. Discussion

Predecessors had done a lot of researches on strawberry virus-free field, and heat treatments in combination with meristem culture also had been reported a lot. However, great differences existed in the reports of different scholars, which could be related to varieties used, experiment time setting and some other external environment factors [33]. Hongqin Miao et al. [34] speculated that high proportion of no disease or light disease strawberry plants could be obtained through shoot tip meristem culture after being treated by heat therapy which could obviously improve the effect of direct shoot tip culture on virus-free. From the results of this study, we could know that the virus-free ratio of constant temperature water bath heat treatment (86.0%) was significantly higher than other treatments and the control group. And the virus-free ratio of constant temperature hot air treatment (50.0%) was not obvious different from control group and had no obvious improvement on the rate of virus-free. It could be inferred that the temperature processing and time setting were not suitable for Arena strawberry, which needed to be further studied.

While Huanle He et al. [22] confirmed that the survival rate of strawberry shoot tip culture with heat treatment was significantly lower than the shoot tip culture without heat treatment, which agreed well with this experiment (the survival rate of inconstant temperature hot air treatment (64.3%) and the constant temperature water bath heat treatment (78.7%) were most close to the control group (89.0%). They were obviously higher than that of the constant temperature hot air treatment (53.3%). When it came to the survival ratio, there were some differences among the three heat treatments, which showed that different heat treatment methods had different extent decrease in the rate of survival. In addition, in this study, the effects of Anna strawberry meristem culture on pollution rate and the number of clustered shoots differentiation had no significant difference among three heat treatments. The results here presented that in the process of actual application, the constant temperature water bath heat treatment would be the most suitable method for Arena strawberry virus-free in the three tested groups.

In the course of this study, we draw a conclusion that the time of starting to turn green for the shoot tips without heat treatment was significantly shorter than the ones combined with heat treatment under the same culture conditions, which was not consistent with the studies of Shanlin Gao [21] and Zhihong Zhang [17], who both reported that all of the shoot tips completed starting to turn green within 3-7 days after vaccination. However, the results of this experiment showed that all shoot tips finish starting to turn green within 14 days. Meanwhile, shoot tips exhibited different degree of browning, and the browning degree of shoot tips combined with heat treatments was particularly serious (constant temperature hot air treatment > inconstant temperature hot air treatment > constant temperature water bath heat treatment > control group). We also had
a further observation about browning. And all data told that the more serious browning happened, the longer time of shoot tips turning green needed. Besides, the time of turning green had a directly negative correlation with the survival rate of the shoot tips. The shorter time of turning green needed, the higher rate of final survival would be. The possible reason that brought about this phenomenon might involve the following aspects: the trial was processed in August, the month when the materials obtained were more than others [35]. And the polyphenols were oxidized more easily in that month. Moreover, the test materials experienced different heat treatments. In addition, the length of operation time and the operation environment temperature might be the external factor leading to browning.

Another finding in this experiment was that the number of clustered shoots differentiation had no significant difference among three heat treatment combinations, though it is significantly lower than the control group. According to relevant documents, the main factors influenced the number of shoot tip clustered shoots differentiation were not only the percentage of hormone in the medium or the length of differentiation time, but also the light of different wavelength [36-41]. It could be inferred from the results that early heat treatments had an even crucial impact on the number of clustered shoots differentiation. Maybe it attributed to the influence of heat treatments on the shoot tips’ endogenous hormones. The specific mechanism under which was not clear.

Different scholars’ views of the reason why heat treatment combined with meristem culture could remove the strawberry viruses were of great difference. In 1953, Posnette [42] pointed out that strawberry viruses could be inactivated by high temperature. There after many other researchers also pointed out that a certain degree of heat treatment could ensure strawberry growth activity but could not completely inactivate all kinds of strawberry viruses. To achieve such a goal, heat treatment must be combined with shoot tip culture [43]. Shuli Li et al. [44] believed that high temperature could deactivate strawberry viruses, and could expand a no virus area at the top of the shoot tip because the spreading of the virus could not catch up with the growth of shoot tip. However, the real mechanism of heat treatment for strawberry virus-free remained to be further tested and verified on molecular level.

References

Effect of Heat Treatment Combined with Shoot Tip Culture on the Virus-Free of Arena Strawberry


Measurement of β-1,3 Glucanase Activity in Permeabilized Discs of Leaves of Healthy and Scald-Diseased Plants

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Abstract: Leaf discs of five cultivars of sugarcane exhibiting different degree of susceptibility to leaf scald were used to measure β-1,3 glucanase activity before and after experimental infection with Xanthomonas albilineans. Leaf discs were permeabilized with iso-propanol to facilitate the uptake of the enzyme substrate by intact tissues and to improve the enzyme assay. Bacterial infection significantly enhances β-1,3 glucanase activity of sensitive cultivars whereas significantly decreased that of the resistant one. Low concentrations of salicylate increase the hydrolase activity whereas jasmonic acid do not act as an elicitor of the enzyme and β-1,3 glucanase, such as laminarin, significantly inhibits the production of β-1,3 glucanase. Thus, the enzyme must be considered as a sensitivity factor induced by the pathogen.

Key words: Elicitors, β-1,3 glucanase, leaf scald, salicylate, jasmonic, sugarcane.

1. Introduction

Plants in general and sugarcane plants in particular, develop different defence mechanisms to combat the invasion of pathogenic organisms. Resistance of plants to disease seems to be a multifactorial process and implies constitutive (structural) and active (biochemical) processes according to their function. Several proteins are involved in these mechanisms by playing a predominant role in the process of resistance against pathogens. Raggi [1] found that the increase of the amount of hydroxyproline-rich glycoproteins in the cell wall of tobacco leaves, produced after their infection by a strain of Y potato virus (PVY), contribute to a general resistance against powdery mildews. The accumulation of these glycoproteins leads to a cell wall toughening and to an increase of peroxidase activity which could stimulate the lignification of the host cell wall. Other proteins, such as some enzymes involved in the pathway of lignin biosynthesis, are involved in the resistance of sugarcane to pathogens [2]. Protease inhibitors are proteins used to regulate proteolytic activities and they act as part of the mechanism of plant defence against the invasion by pathogens. Recently, a protease inhibitor from sugarcane overexpressed in Escherichia coli shows to be able to inhibit the growth of pathogenic sugarcane fungi [3]. An inhibition of the germination of Trichoderma reeseri spores is achieved after the contact between this protein and spores of the filamentous fungus. This protease inhibitor seems to act directly on fungal proteases, impeding the normal development of hyphae. Sugarcane produce glycoproteins, the concentration of which glycoproteins clearly increases after inoculation of plants with smut teliospores. These glycoproteins induce homotypic adhesion and inhibit
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teliospore germination by inhibiting actin capping, which occurs before teliospore germination [4]. Enzymes degrading microorganism walls (chitinases, glucanases), and the production of elicitation compounds (e.g., salicylate, jasmonates), are some active plant biochemical defence mechanisms. In particular, β-1,3 glucanase can be produced by the invaded plant to hydrolyse either glucan components of the cell wall of the fungal invader [5] or glucans detached from its own cell wall by the action of invading bacteria [6].

Leaf scald, a vascular disease of sugarcane, is caused by the bacterium Xanthomonas albilineans (Ashby) Downson. The initial characteristic symptom is a white streak (pencil-line) 1-2 mm wide on the leaf, which follows the direction of the main veins. Later, these symptoms may disappear although plants remain infected. The pathogen is confined to vascular bundles of leaves and stalks which are partially or completely occluded with a gum (a xanthan-like polysaccharide) secreted by the bacterium [7].

In this work, production of systemic plant β-1,3 glucanase and the action of probable elicitors of this defensive proteins are studied as a process of sugarcane plant defence.

2. Materials and Methods

2.1 Plant Material, Infection Conditions and Preparation of Discs Sugarcane Leaves

Saccharum officinarum L. cultivars Louissiana 55-5, Mayari 55-14, Barbados 42-231, Jaronú 60-5 and Cuba 236-5, showing different sensitivity against the pathogen, and field-grown for 8 months, were used throughout this work. Mayari is defined as highly resistant to scald, whereas Jaronú and Louissiana show middle sensitivity and Barbados and Cuba are highly sensitive to scald.

Leaves of sugarcane plants (the third leaf from the top) were inoculated by injecting 0.5 mL of a culture of X. albilineans in the middle rib, maintained for seven days, and then used to assay enzyme activity.

2.2 Assay of β-1,3 Glucanase Activity

Discs (1 cm diameter, 10 discs for reaction) of leaves were permeabilized by incubation on in 16 mL 10 mM sodium phosphate buffer, pH 6.8, containing 0.6 mL 4% (v/v) iso-propanol, for 2 h at 37 °C with continuous shaking. β-1,3 glucanase activity was assayed using commercial laminarin (Sigma Chemical Co.) as a substrate. The complete reaction mixture contained 2 mL of the permeabilization buffer, pH 6.8, ten permeabilized discs of leaves, just obtained, and 1 mL of 0.75% (w/v) laminarin in the same buffer. Reaction was carried out for 4 h at 37 °C with continuous shaking and reducing compounds formed were estimated by using the dinitrosalicylic reagent [8].

2.3 Effect of Potential Elicitors

Discs of leaves were incubated on 15 mL of salicylic acid ranging from 0.2 mM to 1.6 mM in 10 mM sodium phosphate buffer, pH 6.8, containing 0.6 mL of 0.4% iso-propanol for 24 h at 37 °C. Alternatively, discs of leaves were incubated in the same volume of jasmonic acid ranging from 0.02 mM to 0.7 mM or 0.25% (w/v) laminarin in 10 mM sodium phosphate buffer, pH 6.8, containing iso-propanol, for 24 h at 37 °C. After this, discs were used to assay β-1,3 glucanase activity as described. When indicated, leaf discs were floated on the elicitor solution at only one concentration (1.0 mM salicylate or 0.1 mM jasmonic acid), varying then the time of incubation.

3. Results

To improve the method of valuation of the enzyme, leaves of cv. Jaronú 60-5 were chosen since it shows an average sensibility to leaf scald. As it is shown in Fig. 1A, the pre-incubation of leaf discs recently cut in iso-propanol and the development of the reaction in the presence of the permeabilizing agent duplicated the production of reducing sugars after the supply of laminarin as a substrate, indicating that the permeabilization increased twice the access of the polysaccharide to the cellular enzyme. The incubation
Measurement of $\beta$-1,3 Glucanase Activity in Permeabilized Discs of Leaves of Healthy and Scald-Diseased Plants

![Graph A](image1.png)

**Fig. 1** Effect of iso-propanol on cell permeabilization. A: Measurement of $\beta$-1,3 glucanase activity in leaf discs of the sugarcane cv. Jaronú 60-5 in absence or in the presence of the permeabilizing alcohol; B: Time-course of $\beta$-1,3 glucanase activity in permeabilized leaf discs of the sugarcane cv. Jaronú 60-5. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

of discs in iso-propanol for increasing times indicated that already good yields of hydrolysis of laminarin were obtained at 4 h of incubation. Then, this time value was chosen as time of reaction, avoiding longer period of incubation that might involve contamination problems (Fig. 1B).

The assay of $\beta$-1,3 glucanase activity in leaves of the different cultivars ones indicated that the above mentioned activity increases after leaf infection except Mayari, in which significantly decreased. Nevertheless, the degree of increase is according to the cultivar. The major increases of activity after the infection were measured for cv. Louissiana 55-5 (25%) and Barbados 42-231 (20%) for comparison with the activity measured in the controls obtained of healthy plants. Lower increases could be observed for leaves the cv. Cuba 236-51 (7%) and Jaronú 60-5 (11%). Nevertheless, the experimental infection of leaves of the cv. Mayari 55-14 made get down the activity glucanase in 16% with regard to the not infected controls (Fig. 2).

Often, changes of enzymatic activities associated to the entry of the pathogenic agent in the plant are effected by the action of elicitors, of which salicylate and jasmonic acid are the most studied. For the possible action of these elicitors on the activity glucanase of healthy leaves of the cv. Jaronú 60-5, has been tested. Increases of about 38% and 22% were obtained for salicylate concentrations of 0.25 mM and 0.50 mM, respectively, whereas low values of activity were obtained by increasing the concentration of the elicitor, even in cases lower than that obtained ones for control, untreated discs (Fig. 3A). By using jasmonic acid as an elicitor, significant increases of activity were not obtained for the leaves of the cv. for concentrations of the elicitor from 0 to 700 µM (Fig. 3B).

In addition, no significant increases of glucanase activity were obtained varying the time of contact of leaf discs with both elicitors, salicylate (Fig. 4A) and jasmonate (Fig. 4B). The use of laminarin (a $\beta$-1,3 glucan) as a possible elicitor of the enzyme resulted in a significant loss (about 15%) of $\beta$-1,3 glucanase activity after 24 h treatment (data not shown).

![Graph B](image2.png)

**Fig. 2** Measurement of $\beta$-1,3 glucanase activity in permeabilized leaf discs of different cultivars of sugarcane obtained from healthy (white) or scalded (grey) plants. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.
Measurement of β-1,3 Glucanase Activity in Permeabilized Discs of Leaves of Healthy and Scald-Diseased Plants

Fig. 3  Effect of different concentrations of salicylate (A) or jasmonic acid (B) on the β-1,3 glucanase activity measured by using permeabilized leaf discs obtained from Jaronú 50-5 cv of healthy sugarcane. Values are the mean of three replicates. Vertical bars give standard error where larger than the symbols.

4. Discussion

The enzyme β-1,3 glucanase is usually produced by sugarcane leaves and it can be measured in vivo conditions by permeabilizing leaf cells with iso-propanol (Fig. 1). Other alcohols, such as ethanol [9] or methanol [10] have been also used with the same purpose.

Techniques of cell immobilization combined with cell permeabilization are often used to measure enzymatic activities in vivo and in situ and also to improve the secretion of cell products. For example, Stano et al. [11] used Tween 20 to permeabilize glutaraldehyde-immobilized cells of Papaver somniferum in order to facilitate the assay of some amino peptidases. In addition, lactase was also measured by permeabilizing immobilized cells of Populus alba with the same detergent [12]. Hexadecyl trimethyl ammonium bromide and hexadecyl pyridinium chloride provides the best degree of cell permeabilization to assay invertase activity in Cucumis sativus cells although ethanol at variable concentration could also be used [9]. Enzymes involved in the production of fumarprotocetraric acid by Cladonia verticillaris were studied using lichen cells permeabilized with iso-propanol [13] as well as those involved in the biosynthesis of atranorin [14].
Vitality of cells and the permeabilized cell status were preserved for long periods. However, permeabilization treatments tested failed to liberate into the medium arbutin hairy roots of *Brugmansia candida*, able to bioconvert hydroquinone into arbutin, and provoked a total loss in cell viability [15]. For sugarcane leaf discs, iso-propanol seems to be able to improve laminarin uptake by living tissues necessary to achieve the enzymatic reaction in vivo conditions as shown in Fig. 1.

Relative sensitivity of sugarcane cultivars to the scald disease was measured on the basis of the total number of plants and those that exhibited symptoms of leaf scald, such as the white lines parallel to the main vein of the leaves of inoculated plants and re-sprouts, yield per variety estimated as the weight obtained for the total population and brix degree measured with a manual refractometer [16, 17]. The average was recorded as the representative value of the variety. According to these criteria, Louisianna 55-5 and Cuba 236-51 were the most susceptible cultivars whereas Mayari 55-14 was highly resistant to leaf scald. In addition, highest values of β-1,3 glucanase activity correspond to the most sensitive cultivars when diseased, whereas bacterial infection significantly decreases this enzyme activity for the most resistant cultivar (Fig. 2). The enzyme β-1,3 glucanase is in the basis of many antagonist phenomena. *Lysobacter enzymogenes* produces extracellular lytic enzymes capable of degrading the cell walls of fungi and oomycetes. Strain G123, mutated in all three glucanase genes, was significantly reduced in biological control activity against *Bipolaris* leaf spot of tall fescue and *Pythium* damping-off of sugar beet [18]. *Trichoderma harzianum*, a mycoparasite of phytopathogenic fungi, secretes α-1,3 glucanases and showed lytic and antifungal activity against fungal plant pathogens. [19]. *T. harzianum* also produces β-1,3 glucanase the synthesis of which was enhanced by laminarin and polysaccharides contained in the mycelium of *Sclerotium rolfsii*. However, addition of glucose or N-acetyl-D-glucosamine repressed production of this enzyme [20].

As it is shown in Figs. 3 and 4, salicylate seems to be the main elicitor of β-1,3 glucanase of sugarcane leaves at low concentration values (0.2-0.4 mM). Resistance to pathogens and the production of some pathogenesis-related (PR)-proteins can be induced by salicylic acid even in the absence of the pathogenic organism. Salicylic acid is also exported from the primary site of infection to uninfected tissues [21]. Lawton, et al. [22] have been able to characterize transgenic *Arabidopsis* plants that express the bacterial nahG gene encoding salicylate hydroxylase, an enzyme that can metabolise salicylic acid. Strong, constitutive expression of this gene prevents pathogen-induced accumulation of salicylic acid and the activation of systemic acquired resistance [23] by exogenous salicylic acid. However, salicylic acid does not always act as an elicitor of the plant response. Both salicylic and jasmonic acids, whose levels increase on pathogen infection, activate separate sets of genes encoding antimicrobial proteins in *Arabidopsis thaliana* [24]. However, Hause et al. [25] revealed no accumulation of JIP-23, the most abundant jasmonate-inducible protein, in a susceptible barley cultivar attacked by *Erysiphe graminis* f. sp. hordei. Jasmonic acid only produces very low elicitation of β-1,3 glucanase by increasing the time of contact of the elicitor with sugarcane leaf tissues (Fig. 4). Vidal et al. [26] monitored the plant defence gene activation in response to elicitors derived from *E. carotovora* subsp. carotovora by using transgenic tobacco plants. Results showed that mainly pectic enzymes and to some extent one cellulase induce expression of the β-1,3 glucanase gene. Salicylic acid does not appear to be involved in this process.

4. Conclusion

Although β-1,3 glucanase has been considered in general as a resistance protein (RP), glucanase
production by leaf tissue of sugar cane must be seen as a sensitivity protein to the pathogen, *X. albilineans*, since its maximum synthesis corresponds to the maximum degree of sensitivity among the tested varieties. On the contrary, the more tolerant variety decreases the production of the enzyme after infection. It must therefore be concluded that the pathogen elicits glucanase whose substrate is the host cell wall itself.

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Measurement of β-1,3 Glucanase Activity in Permeabilized Discs of Leaves of Healthy and Scald-Diseased Plants


The Allelochemicals Effect of *Zygophyllum album* on Control of *Bromus tectorum*

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**Abstract:** The aim of the present study was to investigate the potential allelopathic effects of different concentrations of *Zygophyllum album* (donor species) aqueous extract (2.5, 5, 7.5 and 10%) on germination percentage, plumule and radicle length of *Bromus tectorum* (weed) and *Triticum aestivum* (crop species) under laboratory conditions to have the greatest inhibitory allelopathic effect on the recipient species in mixed culture compared to that pure culture. The germination percentage, plumule and radicle length of *B. tectorum* in mixed culture was completely inhibited at the highest concentration of aqueous extracts of the donor species level the exerted weak measures as affected by the highest concentration level of donor in pure culture. This inhibition was markedly in obvious *B. tectorum* that is more sensitive to tested donor. The domineering effect of aqueous extract of the donor was more prominent on weeds than crop species. The variant response to the allelopathic substance could be related to the species specific growth regulatory effect of allelochemicals and concentration dependent. There is possibility of using these allelochemicals directly or as structural leads for the discovery and development of environment friendly herbicides to control weeds.

**Key words:** Allelopathy, germination, *Zygophyllum album*, *Bromus tectorum*, *Triticum aestivum*.

1. Introduction

The definition that the term allelopathy refers to any process involving secondary metabolites (allelochemicals) produced by plants, microorganisms, viruses and fungi that influence the growth and development of agricultural and biological systems including positive and negative effects. Allelochemicals from plants are released into the environment by exudation from roots, leaching from stems and leaves or decomposition of plant material [1-3]. Plants or organisms that release these compounds are called “donor species”, while those that are influenced in their growth and development are called “target or recipient species”. Allelopathy includes plant-plant, plant-microorganisms, plant-virus, plant-insects, and plant-soil-plant chemical interactions.

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allelochemicals. Once these substances are identified and characterized, they can be used either as natural herbicides [9]. Medicinal plant had inhibitory effects [10, 11] on selected weeds and its allelochemicals inhibiting weed growth was identified [11]. In addition, the previous results [12, 13] confirmed that it was easier to screen allelopathic plants from medicinal plants than other plants possibly because there existed certain metabolic compounds curing many diseases of mankind in medicinal plants.

The present research is a part of a specific study carrying out in Algeria to explore the allelopathic effects of *Zygophyllum album* on germination percentage, plumule and radicle length of *Bromus tectorum* and *Triticum aestivum* in laboratory condition.

2. Material and Methods

A number of fresh samples from the aerial shoots of the donor species were collected from the natural habitats in the study area during the vegetative stage. The samples were air-dried then after ground in a Wiley Mill to fine uniform texture and stored in glass jars until use. Stock aqueous extract was obtained by soaking 50 g air-dried plant material in 500 mL of cold distilled water (10% w/v) at room temperature (20 ± 2 °C) for 24 hours with occasional shaking. The mixture were filtered through two layers of cheesecloth and centrifuged for 20 min. at 10,000 rpm to remove particulate material and the purified extract was adjusted to pH 6.8 with 1 M HCl. Different concentrations (2.5, 5, 7.5 and 10%) were prepared from the stock solution in addition to the control (distilled water). To achieve this experiment, ten seeds of each of the weed and crop species were arranged in 9 cm diameter Petri-dishes lined with two discs of Whatman No.1 filter paper under normal laboratory conditions with day temperature ranging from 19-22 °C and night temperature from 12-14 °C. Two mL of each level of the donor species extract (2.5, 5, 7.5 and 10%) were added daily to three replicates.

Before sowing, the seeds were surface sterilized with 2% sodium hypochlorite for 2 minutes then rinsed four times with distilled water. The sterilized seeds were soaked in aerated distilled water for 24 hours. The experiment includes three seed sowing treatments 1-weed only (weed pure culture), 2-wheat and weed (mixed culture) germination percentage (GP), inhibition percentage (IP), and plumule (PL) and radicle length (RL) were recorded after one week at the end of the experiment. Seed germination index (SGI) was calculated according to the following equation [14, 15].

\[
\text{SGI} = \frac{\sum T_i N_i}{S}
\]

Where, \(T_i = \) the number of days after sowing; \(N_i = \) the number of seeds germinated on day \(I\); \(S = \) the total number of seeds planted.

Relative reduction or stimulation of seed germination and radicle length as affected by the allelopathic substance were calculated according to the general equations:

\[
\text{Inhibition percentage} = \left[1 - \frac{\text{allelopathic}}{\text{control}}\right] \times 100
\]

Statistical analysis: data of the present study were subjected to standard one-way analysis of variance (ANOVA) using the COSTAT 2.00 statistical analysis software manufactured by CoHort Software Company (1986).

3. Results

Data of germination percentage (GP), seed germination index (SGI), germination inhibition percentage (GIP), plumule (PL) and radicle (RL) length of the two weed species and wheat beside their statistical representation are illustrated in Table 1.

Commonly, GP of *B. tectorum* in pure (B) and mixed cultures (B×W) was significantly \((P \leq 0.05)\) affected upon applying different concentrations of *Z. album* aqueous extract (ZAAE) (Table 1 and Fig. 1). It was obvious that in *B. tectorum* pure culture, the value was about 100% at control level. Continuously, it was decreased to about 83.3% at 2.5% and 5% ZAAE concentrations. A great noteworthy
The Allelochemicals Effect of *Zygophyllum album* on Control of *Bromus tectorum*

**Table 1** The effect of different concentration of *Z. album* aqueous extract (ZAAE) on germination efficiency of *B. tectorum*.

<table>
<thead>
<tr>
<th>Variables treatment (%)</th>
<th>GP (%)</th>
<th>SGI</th>
<th>GIP (%)</th>
<th>PL (mm)</th>
<th>RL (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>B=W</td>
<td></td>
<td>B</td>
<td>B=W</td>
</tr>
<tr>
<td>C</td>
<td>100.0a</td>
<td>96.6a</td>
<td>32.33a</td>
<td>29.70a</td>
<td>19.66a</td>
</tr>
<tr>
<td>2.5</td>
<td>83.3b</td>
<td>75.0b</td>
<td>23.49b</td>
<td>13.05b</td>
<td>11.66b</td>
</tr>
<tr>
<td>5.0</td>
<td>83.3b</td>
<td>20.0c</td>
<td>19.6c</td>
<td>5.00c</td>
<td>1.66c</td>
</tr>
<tr>
<td>7.5</td>
<td>26.6c</td>
<td>0.0d</td>
<td>4.67d</td>
<td>0.00d</td>
<td>0.00d</td>
</tr>
<tr>
<td>10.0</td>
<td>20.0d</td>
<td>0.0d</td>
<td>3.42d</td>
<td>0.00d</td>
<td>0.00d</td>
</tr>
</tbody>
</table>

*P*-value 0.041* 0.018* 0.049* 0.15 0.39

Two-way ANOVA

| A-treatment | ** | ** | ** | ** | ** |
| B-seed culture | ** | ** | ** | ** | ** |
| AB interaction | ** | ** | ** | ** | ** |

Different letters within each column indicate significance at *P* < 0.05; *: significant at *P* < 0.05 as evaluated by *t*-test; Two-way ANOVA: NS: not significant; *: significant at 0.05; **: significant at 0.01.

**Fig. 1** The different effect of *Z. album* aqueous extract (ZAAE) on germination efficiency in petri-dish experiment.

Reduction in GP values was attained along the higher ZAAE concentrations. Correspondingly, values were decreased to about 26.6% and 20% at 7.5% and 10% ZAAE concentrations, respectively. Lower values were detected in mixed culture compared to that estimated in pure culture. At control level, the value was initiated at about 96.6% and reduced to 75% at 2.5% concentration. Continuously, at 5% ZAAE concentrations, a great inhibition of about 20% respectively has occurred. Finally, germination was completely inhibited at 7.5% and 10% ZAAE concentration.

With respect to SGI of *B. tectorum* the value decreased distinctly as ZAAE concentration increased in pure and mixed culture. This reduction was statistically (*P* ≤ 0.01) highly significant. Starting with pure culture, SGI began with a value of about 32.33 at both controls. On the other hand, lower SGI value (29.7) was detected in mixed culture at control. Continuously, in pure culture, the values 23.49 and 19.6 were obtained at 2.5% and 5% ZAAE concentrations, while in mixed culture the two values were reduced to 13.05 and 5, respectively. Finally, SGI declined till reached to the minimum values (4.67 and 3.42) in pure culture at 7.5% and 10% ZAAE concentrations, respectively,
and on the other hand, the zero values at 7.5 and 10% ZAAE concentrations (Table 1 and Fig. 1).

In completion, data of the present study also demonstrated that GIP of *B. tectorum* was significantly affected \((P \leq 0.01)\) due to the apparent allelopathic action of ZAAE concentrations in both pure and mixed culture (Table 1 and Fig. 1). Not any GIP was attained at controls. Alternatively, at 2.5% concentration level, GIP in pure culture was (16.7%) compared to that attained in mixed culture (22.36%). To go through with this, GIP attained values of about 16.7%, 73.4% and 80% at 5%, 7.5% and 10% ZAAE concentration, respectively, in pure culture compared with 79.29%, 100% and 100% in mixed one.

In *Bromus tectorum* pure culture, the plumule elongation was not completely inhibited by the extract, but it was less at higher concentration levels (Table 1 and Fig. 1). Obviously, all allelopathic concentrations have reduced PL. Statistically, the applied concentrations of ZAAE, type of seed culture and their interaction were significantly \((P \leq 0.05)\) affecting PL. As well, the immense negative response of the plumule growth was marked at 7.5% and 10% concentrations in both pure and mixed cultures. Actually, at control level, PL of *B. tectorum* was about 19.66 and 25 mm in pure and mixed culture respectively. On the other hand, 2.5% and 5% concentrations were considered as an inhibited concentration (the values in pure culture were about 11.66 and 1.66 respectively). On the other hand, in mixed culture, PL at 5%, 7.5% and 10% concentration levels was completely inhibited.

Compared to control, a gradual decrease in RL of *B. tectorum* was observed along the gradual increase in ZAAE concentrations in pure and mixed cultures (Table 1 and Fig. 1). RL implication was significantly affected by the treatment at \(P \leq 0.01\), their interaction are significantly affected at \(P \leq 0.05\) while the type of seed culture consequence was not significant. At control, the values of RL were 34 and 39.33 mm in pure and mixed culture, respectively. Higher concentrations of ZAAE were notably active disturbing radicle emergence. In pure culture, and at 2.5%, and 5% concentrations, RL decreased to 14.33 and 9.33 mm. Constantly, it continued reduction till it attained a value of about 2.83 and 2.33 mm at 7.5% and 10% concentration level. Almost the same reduction has occurred in mixed culture; the lowest value (zero mm) of RL was noticed at 7.5% and 10% ZAAE concentrations. At 2.5% and 5% ZAAE concentrations, RL decreased to 16 and 5 mm respectively in mixed culture.

4. Discussion
The present work was carried out as a preliminerly study to investigate any possible herbicidal activity of the selected species against widely spread weed. The allelopathic effect of 2.5%, 5%, 7.5% and 10% aqueous extract beside the control from aerial shoots of *Z. album* was clearly demonstrated on germination percentage, plumule and radicle length of *B. tectorum* in mixed culture. Considering the foregoing results, it seemed that there are significant phototoxic effect of *Z. album* on germination and plumule and radicle length. These results correlated with the findings that allelochemicals presented in the aqueous extracts of different plant species have been reported to affect different physiological processes through their effects on enzymes responsible for phytohormone synthesis and were found to associate with inhibition of nutrients and ion absorption by affecting plasma membrane permeability [16]. *Z. album* species have phytotoxic effect on germination and plumule and radicle length of *B. tectorum* the germination and plumule and radical length were sensitive to the increasing concentration of the aqueous extract.

The aqueous extract of the donor plants showed a wide range of activities from partial and complete inhibition to stimulation which may indicate the presence of certain allelochemicals causing inhibition [17, 18]. Zzet and Yusuf [7] stated that, plant directly affect another plant either positively or negatively through exuding chemical substances.
Some species under the present study exhibited a stimulatory effect upon the recipient species which may be through hormonal activities or promoting growth through adequate mineral supply. Other workers indicated that the effect of a given compound or plant metabolites may be inhibitory or stimulatory depending on their concentrations in the surrounding medium [19, 20].

Phytochemical screening of *Zygophyllum album* revealed the presence of alkaloids, flavonoids and saponins as major components, as well as the presence of carbohydrates and/or glycosides, coumarins, sterols and/or triterpenes, tannins and cardiac glycosides [21]. One of the most prominent results in this work is that extracts are more harmful to weeds extracts, which may be due to the presence of allelochemicals, such as alkaloids, amino acids, carbohydrates and phytohormones at higher concentrations in shoots [22].

References


Evaluation of Biomass Supply Chain from *Robinia pseudoacacia* L. SRF Plantations on Abandoned Lands

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**Abstract:** Bio-energy and biomass system is a new scientific field, very interesting, where there can find new available information; however, there is also place for other novelties. A lot of projects and experiences regarding to what mentioned above, lead to the credence that forest biomass assume a fundamental role, inasmuch they constitute a renewable resource, already or easily present, in the environment. The main purpose of undertaking this study is to analyse the obtainable biomass yields from black locust (*Robinia pseudoacacia* L.) SRF. Four different plantations have been situated on lands of different fertilities in Italy and Albania. Neither irrigation nor fertilization has been applied. Two different methodologies of biennial harvesting have been compared on black locust plantation situated in Italy. The first one is the contemporary cutting and chipping, while the second with cutting, field seasoning of trees and then chipping. In Albania the analyses of planted trees were mainly focused on height and diameter growth. They revealed a good continuation which means that the bed conditions of abandoned terrain, and climate considering the extended drought period of last summer, don’t affect the growth.

**Key words:** Biomass, growth variation, height increment, diameter increment, implant spaces, mean weight, humidity.

**1. Introduction**

Ligno-cellulosic biomass, wood in particular, is the most ancient and diffused energetic source. Bio-energy or “biomass system”, replacing fossil combustible even though partially, generates positive effects regarding to atmospheric pollution, soil protection, rural progress, and employment. Firewood is traditionally utilized for heat production in stoves, fireplaces, furnace etc. For the same purpose, nowadays is more preferable either in industry or in habitations the chipped wood or pellets as they are of easy clipping.

For electric energy production, biomass normally adaptable to combustion, has been utilized, which means a C/N rapport superior to 30, and inferior to 40% humidity [1].

Whole plant harvesting, and border stack establishment, allows to profit by natural exsiccation of the entire plant in open air, and to obtain a dryer chipped combustible, outsold at more suitable price than a fresh one.

Species being considered for short rotation forestry included willows in north and west Europe, black locust in the Mediterranean region and eucalyptus in the Iberian region and southern France [2].

Usually the black locust rotation turns vary from five to 15 years. The black locust wood is considered a suitable energetic source, for the elevated calorific power. Several tests have evidenced values equal to 18.2-19.8 kJ/kg. Restrained wood humidity average of 31.6% (fresh weight), has ensued inferior to that of other broadleaves trees, and little variable due to environment Societa Agricola e Forestale (SAF) [3].
2. Materials and Methods

The study was organized in four different sites in two countries of Italy and Albania, respectively: Monte Romano (Italy), Azienda Agraria Della Tuscia (Italy) (The Nursery of the University), Montalto (Italy) and Valias (Albania).

The obtainable woody production (fresh and dry biomass) measured in ton in Montalto, was determined through weighing of 100 trees.

\[
P = \frac{W}{1000} \times N \quad \text{(Ton·ha}^{-1} \text{in two years)} \quad (1)
\]

where \( P \): fresh biomass productivity in two years; \( W \): mean weight value of two-year plants (kg); \( N \): plants number/ha.

\[
P_0 = \frac{P}{2} \times (1 - h) \quad \text{(Ton·ha}^{-1}/\text{year)} \quad (2)
\]

where \( P_0 \): dry biomass productivity; \( h \): humidity.

The moisture content of trees at cutting time was determined through sample discs collected from 30 trees.

\[
u = \frac{M_u - M_a}{M_a} \times 100 \quad (3)
\]

where \( u \): humidity percentage; \( M_u \): fresh weight; \( M_a \): anhydrous weight.

In experimental area of Monte Romano, diameter and height surveys have been made from April 2007 until April 2008. In Azienda Agraria (University Nursery), the same surveys were started at April 2007 and finished at December of the same year.

The height and diameter growth surveys of these plants have been effectuated after plantation.

2.1 Experimental Area of Monte Romano (Italy)

Monte Romano extends to latitude of 42°16′3"00 N, and a longitude of 11°53′40"92. The minimum and maximum heights above the sea level are 27 m and 370 m, respectively.

It is characterized by a climate with annual rainfall ranging from 810 to 1,519 mm, a dry summer reduced to two or three months and an average temperature of the coldest month of the minimum at around 2.3-4 °C, and an average maximum temperature of the hottest month around 24.5 °C (Fig. 1).

The soil type is clayey-arenaceous flysch marl, these are low permeability soils. Volcanic soils, which are more permeable, can also be found. Establishment in the experimental area of Monte Romano has been done by seedlings, already rooted, gently conceded from Forest Service of Pieve Santo Stefano nursery.

No survey has been achieved prior to plants plantation. Implant spaces applied has been 1 m × 1 m.

The collected data have been elaborated using Excel program. Standard deviation has been determined for all the data.

2.2 Experimental Area of Azienda Agraria Della Tuscia (University Nursery) (Italy)

The site is located in the town of Viterbo, which extends to latitude of 42°25′7″ and longitude 12°6′34″. The minimum and maximum heights above the sea level of Viterbo are 86 m and 896 m, respectively.

The climate of this area is characterized by abundant rainfall generally up to 1,614 mm, the dry

![Fig. 1 Bagnouls-Gaussen diagram.](image-url)
Evaluation of Biomass Supply Chain from *Robinia pseudoacacia* L. SRF Plantations on Abandoned Lands

Summer is absent or poorly marked, while the average minimum temperature of the coldest month is usually lower than 0 °C.

The lands that make up this area are mainly of volcanic origin and only towards the coast are represented by recent clay deposits.

Even in this occasion seedlings already rooted, conceded from Forest Service of Pieve Santo Stefano nursery, have been exploited. This time the terrain was not as rough as in Monte Romano.

Prior to plantation the parcel terrain was mechanically treated with tractor.

The same 1 m × 1 m implant spaces and the same surveys of height and diameter growth have been effectuated.

2.3 Experimental Area of Valias (Albania)

The experimental area of area Valias extends between the latitude 41.33° N and longitude 19.82° E.

The hottest month is August with temperatures ranging from 17 to 31 °C. In January, this is also the coldest month, temperatures ranges from 2 to 12 °C. November which is also the wettest month has an average rainfall of 211 mm, while the driest period in July and August has an average rainfall of 32 mm. The average annual rainfall is 800-1,100 mm (Fig. 2).

The soils of this area are mainly those brown that have a profile with different genetic horizons. O horizon is absent. The layer of humus is dense enough about 30-40 cm with a gray-brown or light brown color. The percentage of humus content is of 4-9%.

The seeds of black locust used on this experimental area have been treated in the warm water. First the seeds were planted in lines and after the first year they have been transplanted in the same area but this time using 1 m × 1 m spaces. The irrigation has been applied the terrain conditions this time were much better than in the other two cases, for a long period, especially during the summer, and even in this experimental area the same surveys have been applied.

2.4 Experimental Area of Montalto (Italy)

The area of Montalto lies at a latitude of 42°21'5", and a longitude of 11°36'28". The minimum and maximum heights above sea level are 0 m and 101 m.

The climate here is characterized by hot-arid climatic conditions, ranging from the Mediterranean conditions characterized by annual rainfall of 649 mm, with five months of dry summer and the minimum average temperature of the coldest month of 8.3°C, to the mixed deciduous oaks climate, with an annual rainfall of 1,133 mm, four-month summer drought and the minimum average temperature of the coldest month of about 4 °C (Fig. 3).

In this area we find the presence of the sandy and sandy-conglomeratic overlay.

In this study, two black locust plantations in central Italy were considered with biennial cutting cycle.

The plantations were situated in two different fertility
Evaluation of Biomass Supply Chain from *Robinia pseudoacacia* L. SRF Plantations on Abandoned Lands

and climatic zones, on lands abandoned by traditional agriculture. The first plantation (low yield) was situated in a sand-rocky soil, with scarce fertility, annual precipitation of 600 mm and summer aridity. The second (high yield) was situated in a clay-silt soil with medium fertility and annual precipitation of 700 mm. The previous cultivation was wheat in the first case and vineyard in the latter. The plantations density was of 3,333 trees·ha$^{-1}$, a twenty-year productive cycle was hypothesized and the release of stumps for the production of firewood at the end of biomass production. These plantations were characterized by a low input level, irrigations or fertilizations were not performed, since the rusticity of black locust and its resistance to the summer aridity [4]. The obtainable woody production (fresh and dry biomass) in ton was determined through the weighing of 100 trees, collected in the two years old plantations. The moisture content of trees at cutting time was determined through sample discs collected from 30 trees; sample were dried in stove (UNI 9091/2), this measure was repeated after three months of field seasoning (Figs. 4 and 5).

The fresh weight and the moisture content (wet basis) determined obtainable dried biomass per hectare.

3. Results

In experimental area of Monte Romano the survival of black locust to the terrain conditions were nearly 90%. The annual increment respectively in diameter and height were 0.51 cm and 54 cm as shown in Figs. 6 and 7.

In Azienda Agraria (University Nursery), if we compare the growth of black locust to that of Monte Romano, we will see clearly that in this experimental area black locust grows better because of the better terrain. The survival percentage of these plants was 100%.

The annual increment respectively in diameter and height were 0.59 cm and 84.6 cm (Figs. 8 and 9).
Fig. 8  Average diameter (cm) of robinia in nursery.

Fig. 9  Average height (cm) of robinia in nursery.

The height and diameter growth of black locust planted in the experimental area of Valias were higher than in both two other cases, not only for the better terrain condition but also for the irrigation during the summer period influenced this. The growth values that resulted from the surveys are reflected in Figs. 10 and 11.

The mean diameter and the mean height value of black locust plantation in Montalto were 3.8 cm with a standard deviation of ± 1.40, and 269.5 ± 0.53 cm, respectively. Mean increment in each year (first and second) of diameter and height were 1.9 cm and 134.75 cm, respectively.

Fig. 10  Average diameter (cm) of robinia in Valias (Albania).

Fig. 11  Average height (cm) of robinia in Valias (Albania).

The mean stem weight was 1.639 kg, and the mean branches weight was 1.307 kg for a total mean weight (stem and branches) of 2.946 kg. Branches influence in the total weight was about 44% (Table 1).

The mean density of black locust plantation in Montalto is 931 kg·m⁻³ while the mean basic density is 549 kg·m⁻³. Bark percentage is nearly 30% and humidity is nearly 60% (Table 2).

Base-middle-top: As it has been mentioned above, three samples for each stem have been analysed. These samples were situated one in the base of the stem, the

<table>
<thead>
<tr>
<th>Implant density, plants·ha⁻¹</th>
<th>Mean basic diameter, cm</th>
<th>Mean height, m</th>
<th>a, kg</th>
<th>b, %</th>
<th>c, kg</th>
<th>d, %</th>
<th>e, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>3333</td>
<td>3.7 ± 1.40</td>
<td>2.70 ± 0.53</td>
<td>1.64</td>
<td>29</td>
<td>43.98</td>
<td>40</td>
<td>2.95</td>
</tr>
</tbody>
</table>

a: Average fresh weight of the stem, kg; b: Bark percentage on the stem fresh weight, %; c: Average weight of the branches of the plant, kg & %; d: Bark percentage on the fresh weight of branches, %; e: Total plant average weight, kg.

<table>
<thead>
<tr>
<th>Sample volume, cm³</th>
<th>Density, g/cm³</th>
<th>Basic density, g/cm³</th>
<th>Bark percentage, %</th>
<th>Humidity percentage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>Middle</td>
<td>Top</td>
<td>Base</td>
<td>Middle</td>
</tr>
<tr>
<td>52.6</td>
<td>15.7</td>
<td>4.9</td>
<td>0.999</td>
<td>1.021</td>
</tr>
</tbody>
</table>
Evaluation of Biomass Supply Chain from *Robinia pseudoacacia* L. SRF Plantations on Abandoned Lands

Basic density permits us to have an important indication of wood energetic contents. Basic density value is always referred to the dry wood. It is necessary to distinguish the quality of wood material, based on its composition (Figs. 14 and 15).

It is also important to know the bark percentage, considering the fact that the bark is supposed to have a higher calorific power than the wood.

The comparison of main xylo-energetic characteristics between two different methodologies of biennial harvesting on black locust plantation is shown on the following graph. The first methodology is the contemporary cutting and chipping, while the second with cutting, field seasoning of trees and then chipping (Fig. 16).

This collected data have been used to calculate the obtainable productivity from this implantation. The plant has been harvested every two years, and the biomass quantity for this period was:

\[
P = \frac{W}{1000} \times N \quad \text{(Ton·ha}^{-1} \text{in two years)} \quad (1)
\]

where \(P\): fresh biomass productivity in two years; \(W\): mean weight value of two-year plants (kg); \(N\): plants number/ha.

\[
P_0 = \frac{P}{2} \times (1 - h) \quad \text{(Ton·ha}^{-1} / \text{year)} \quad (2)
\]

where \(P_0\): dry biomass productivity; \(h\): humidity.

### 4. Discussions

According to Keresztesi [5], black locust grows
rapidly, especially when young. Trees can reach 3 m tall in one growing season and average 0.5-1.5 m height and 0.2-2 cm diameter growth per year. Roach [6] also reported that those black locust seedlings grow rapidly when planted on good sites and competition is sparse. Average annual height growth of five-year-old plantations ranged from 0.3 m on severely sheet-eroded sites to 0.8 m on sites with little or no erosion. The growth of our black locust in University Nursery is within the range reported by Keresztesi [7].

Diameter growth is 0.57 cm per year, and the height growth is 54.28 cm. If we compare this data to those of Monte Romano when the terrain was worse, the diameter growth is 0.34 cm per year and height growth is 14.98 cm. This case can be considered similar to Roach [6] about sheet-eroded sites.

About physic characteristics of black locust wood in Montalto, comparing the data found out from the study and the data reported by the literature we can say:

Giordano [8] reported that the black locust density of 750 kg/m³ when the humidity value is 12%, while our average density is of 931 kg/m³ when the average humidity value is 59%. This means that in 1 m³ there are 931 kg wood, and 59% of this quantity is water. Zilli [9] reported that the values of black locust density were closer to our values. He reported the value of 1,050 kg/m³, when the humidity is of 54%. The density diminishes on the top of the plant because of non-lignified material rich in water. As it is known the lower the humidity the higher the calorific power of specie.

Our experiment furnishes different values of basic density varying on the sample position in the plant, diminishing from the bottom to the top. Our mean basic density value is of 549 kg/m³. Zilli [9] reported that the black locust basic density value was 680 kg/m³. This is an indicator of diverse lignifications and as a consequence an indicator of different energetic contents also.

Our experiment reports the average black locust bark percentage which is 29%, lower on the plant’s base and higher on the top.

5. Conclusions

The acquaintance with the experiments results, situated in four different plots, announce that black locust grows fast and without special requirements about climate, terrain, etc.

Therefore, after the experiments, the outcome has designated, black locust is the fastest growth species. This is the reason why it needs a special attention, to be widely diffused in the future, especially in the abandoned and degraded areas. Black locust as pioneer specie helps to ameliorate the conditions of these areas enriching the soil with nitrogen and can be used as a renewable energetic resource to reduce as much as possible the energetic problems of nowadays.

Then ahead valuable forest species can be situated instead of black locust. Significant species role is also of erosion and landslide protection of emplacement surface.

The obtainable fresh biomass productivity in two years was 6.66 t/ha. Dry biomass productivity in one year was 1.96 t·ha⁻¹/year. Considering this values black locust can replace the agricultural species that yields less in these terrains.

References

Potential Effects of Global Change to Urban Vegetation: Vulnerability and Adaptations

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Abstract: An urban area is a space with high population density which develops new, major and complex structures in comparison to the areas surrounding it. In order to develop these structures and maintain population and its activity, the metabolism of urban areas needs a lot of external sources of energy and nutrients (water, food, materials...), which produces heat waste, garbage, sewage and pollution which are some of the major problems for urban sites, and the related areas from it. This metabolism promotes major environmental changes in the urban areas, which promote stress on vegetation used in gardening. The main environmental factors that affect vegetation in urban areas are the same that have been defined in literature from long time ago, but now they are acting as the sum of complementary and synergic effects of these classical stresses at the same moment, in the same place, which happen due to the incredibly amount of energy that we place in the systems. This is called global change. Ecophysiological studies can provide objective information to be used as a tool to improve the vegetation management in urban areas from design to process, and consequently avoiding the potential vulnerabilities associated with global change. Present paper tries to show several examples about the plant response, measurement tools and vulnerabilities and adaptations to global change under urban conditions. It can be concluded that the large availability of vegetal material and the great technical development can be highlighted as strong points of gardening and urban landscaping while, as weak points, it could be mentioned the changing taste of consumers, which can force the introduction of new vegetal material with no time for adaptation. Urban gardening and landscaping can be considered to be exposed to global change, but in our opinion it is necessary to carry out more studies to determine the real degree of vulnerability of this activity to this complex kind of stress.

Key words: Ecophysiology, water-use efficiency, plant water relations, biotic and abiotic stresses.

1. Introduction

Already in the Book of Psalms (48.2) appeared the term “landscape” is maintained in romantic literature of XIX century and it refers mainly to an aesthetic, geographical and to some extent dynamic bit concept. Later, the concept of “landscape ecology”, the landscape as the visual representation of an intricate network of biodiversity appeared (Fig. 1). Recently, it was developed the term “restoration of the landscape” which addresses the need to put the landscape back to its original state of diversity after it has undergone some types of disturbance. This can be done in a natural manner or with the help of techniques and systems [1, 2].

Designers of cities and landscapes generally make plans and perform their work before the agricultural engineers, gardeners, ecologists, physiologists, which causes imbalances and environmental problems for the management of urban green. An urban area is a space with high population density which develops new, major and complex structures in comparison to the surrounding areas. In order to develop these structures and maintain the population and its activity, metabolism of urban areas needs a lot of external sources of energy and nutrients (water, food, materials, etc.) and it produces heat, waste garbage, sewage and pollution. This metabolism develops specific microclimates, which are attributable to the large clustering of heat absorbent surfaces that heat up under
sunlight, the important modifications in hydrological cycle due to drastic soil reduction and the channeling of rainwater into underground ducts. This metabolism promotes major environmental changes in the urban areas.

Since XIX century, the hygienist movement has developed a new way of life by means of gardens and landscape design. This process has been increased in the last decades together with the development of social economics and social sensibility. As a result, urbanism and landscaping have acquired a very important role in the quality of life.

Barcelona must be used as example of effects of environmental conditions on vegetation. It’s an old city located on the Mediterranean coast in the Iberian Peninsula, with a typical Mediterranean climate (Fig. 2). The City of Barcelona has about 60 different types of green areas, including historical parks, thematic parks and forests (Table 1).

Fig. 1 Landscape representations as a biodiversity mesh from Zonneveld (1989). Similarly to other ecosystems, a city is a complex ecosystem and consequently it must be studied according ecophysiological science and tools [3].

Fig. 2 Climatic diagram of Barcelona city.
Table 1  Characteristics of Barcelona’s green areas in 2007 (àrea medi ambient ajuntament de Barcelona).

<table>
<thead>
<tr>
<th>Category</th>
<th>Area (ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urban green (urban parks, gardens, trees in streets)</td>
<td>1,040</td>
</tr>
<tr>
<td>Forest green (Collserola Serra)</td>
<td>1,795</td>
</tr>
<tr>
<td>TOTAL</td>
<td>2,835</td>
</tr>
<tr>
<td>Green per person (with Collserola area)</td>
<td>18</td>
</tr>
<tr>
<td>Green per person (without Collserola area)</td>
<td>7</td>
</tr>
</tbody>
</table>

The potential climate change attributable to global change can increase local and general temperature [4]. These yearly small changes in temperature may have great influence in the atmospheric carbon balance [5]. This increase will not be the same around the world [4]; it seems to be more pronounced in the Mediterranean basin [6]. According to the most pessimistic predictions, the air temperature in this area may increase up to 4 °C and the average annual rainfall may have a 10 to 40% decrease in the last years of XXI century [7].

Mediterranean environments are characterized by a double stress [8]. In summer, low soil hydric availability, together with high vapour pressure deficits at atmospheric level bring inhibitions in plants growth and different negative effects in their development, as changes in the form and size of leaves and canopies, in phenolgy, in biomass distribution, etc. [3]. Despite the value of the different components of global change (soil uses, climate change, population distribution, invasiveness, etc.), the really important, it’s their integral, drought.

Predictions arising from different models for generating climate change scenarios showed as the Central American and Mediterranean regions would be affected by drought periods of half length (4-6 months) and long (more than 12 months), which will be three and eight times more frequent than at present [9].

Plants are affected by stress, but not all are vulnerable to it. The most important stresses [10] are:

- **Abiotics:** drought, flooding, salinity, high, low, chilling and freezing temperatures, high radiation, ozone, mineral deficiencies, etc.
- **Biotics:** insects, fungi, bacteria, viruses, elicitors, and competition among species.
- **Anthropogenics:** air (O3, NOx, SO2, aerosols), water (salinity, microbiology, heavy metals, drugs) and soil (heavy metals, structure loss) pollution, herbicides, acid rain, dry deposition, tourism, etc.

Global change is the combination of many of them in the same space and at the same time, which can cause synergic effects on vegetation, on crops, on gardens, etc.

The factors that play an important role in the productivity of gardening and landscaping are:

- The increase of temperature that produces an increase in the evapotranspiration (ETP), in the soil respiration and in the amount of organic matter, reduces soil water capacity [11].
- The increase of CO2 concentration must increase productivity and the efficient use of water, therefore, plants develop photosynthesis regulation and productivity returns to the original values or to inferior ones [12].
- The increase in ultraviolet (UV) radiation produces important morphological, physiological and biochemical changes on vegetation. Despite the negative effects on growth, this stress causes the increase of flavonoids and some antioxidants biosynthesis [13].
- Drought causes growth reduction but, in general, in the Mediterranean area, this appears together with other stresses, and the effects can be modified by the interactions [3].

Environmental stresses are the main cause of yield depletion. The current crops and gardens are reduced from 3 to 7 times with regard to their potential from 3 to 7 times with regard to their potential productivity.

Abiotic stresses and weeds represent 90% of this reduction, illnesses 6% and insects 4% [14]. The main characteristic of green plants is the assimilation of CO2 [15], all the other physiological, morphological and metabolic characteristics (vacuole, cuticle...) are secondary [16, 17].

Plants need to keep their stomas open in very dry environments, consequently, they continuously lose
water (transpiration) and a continuous water flow is established between the soil and the atmosphere [18].

All environmental conditions promote hydric deficits in tissues cause stress, which describes adverse environmental conditions for normal growth. These conditions, particularly their combination in a short time, can cause important stresses to plants and to gardens.

With the present conditions and the potential future ones, the following solutions can be considered in order to avoid or reduce plant vulnerability to global change under urban conditions:

- Adapting vegetal material to its designated location and expected use, considering its ecophysiological characteristics.
- Improving soil water storage and fertility.
- Increasing water-use efficiency. This could be developed by means of methods and systems that integrate our needs, as users, with plants and water availability:
  - Sensors, to help in agronomical decision making;
  - Regenerated water for irrigation.

2. Material and Methods

Due to this paper has been prepared and developed as invited conference of ISHS 2nd International Conference on Landscape and Urban Horticulture (Bologna, Italy, 9-13th June of 2009), material and methods has not been developed and reader can find all the specifications in literature.

3. Results

Following reports some examples of these proposed options, potentially solutions. Fig. 3 shows the study of limits of plant survival in order to improve and adapt agronomical options, so irrigation must be restricted and or minimum irrigation technique could be applied until some physiological status, below this value is difficult aesthetic requirements disappear and plant survival is difficult [19].

Table 2 points out the selection of different plant species for their morphological traits to maintain the bigger number of insect depredators in order to help integrated pest management in gardens and landscape restorations. These traits are showing as cuticle, hairs and osmotic adjustment that can play an important role in the relationship plat/host, and consequently these plant characteristic must be used in integrated pest management [20].

Soil volume restriction in parks of urban areas promotes important reductions in growth (Fig. 4), which could be partially attributable to the root system constriction and to the lack of water and nutrient sources due to small volume and/or substrate with poor physical characteristics. These deleterious effects on plant growth would be increased with plat edge, above all when the vegetation is old [21].

<table>
<thead>
<tr>
<th>Predators population level maintenance</th>
<th>Plant species</th>
<th>Ecophysiological characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>++++</td>
<td>Ononis natrix</td>
<td>High moisture content in tissues</td>
</tr>
<tr>
<td>+++</td>
<td>Inula viscosa</td>
<td>Non-glandular foliar hairs, low density of hairs and thin cuticles</td>
</tr>
<tr>
<td>++</td>
<td>Cistus monspeliensis</td>
<td>Very xeric plant</td>
</tr>
<tr>
<td>+</td>
<td>Erigeron karvinskianus</td>
<td>Thin cuticles, low water content in drought, non-glandular hairs</td>
</tr>
</tbody>
</table>

Fig. 3  Limitations to hardening: effects of maximum stress in the recovery of Quercus coccifera [19].
Potential Effects of Global Change to Urban Vegetation: Vulnerability and Adaptations

4. Conclusions

In order to maintain a correct management of the urban vegetation, it’s important to take into consideration the global change, which is the sum of complementary and synergic effects of these classical stresses at the same moment, in the same place, which happen due to the incredibly amount of energy that we place in the systems [8, 23].

So, it can be concluded that the large availability of vegetal materials and the great technical development can be highlighted as strong points of gardening and landscaping while, as weak points, we could mention the changing taste of consumers, which can force the Mediterranean conditions (Fig. 6), but several chemical and microbiological aspects must be taken into consideration to maintain a good ornamental value of vegetation and a high health security level for people [21].

The use of sensors, mainly optical sensors can provide objective and practical tool that permit the detection of plant status by remote sensing and help to take agronomical decisions about it (Fig. 5). Digital cameras beside of these options are chip and easily to use [22].

The use of regenerated water is and will be an important alternative to irrigate parks especially under
introduction of new vegetal material with no time for adaptation. Gardening and landscaping can be considered to be exposed to global change, but in our opinion it is necessary to carry out more studies to determine the real degree of vulnerability of this activity to this kind of stress.

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Particular Ways of Planting in Cemeteries of Šiauliai City (Lithuania)

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Abstract: The issue of cemetery planting is not yet sufficiently studied in Lithuania. Cemetery is one of the sites where people could give way to their subjective comprehension of beauty. Analysis of the cemetery planting revealed certain noticeable features that could be ascribed to the forms of folk art. These are various objects with implied specific content or ornamented compositions. The aim of the research was to ascertain and distinguish graves characterised by specific planting features associated with plants, their layouts on graves. Analysis of the research results shows that two options form the peculiarities of grave planting: depiction of specific objects and ornamentation. We think that majority of the ornaments are made with the single purpose of decoration, and do not associate with symbolic meanings. During this research the graves decorated with planted symbolic objects and ornaments were distinguished. This phenomenon becomes more and more popular in cemeteries of Šiauliai city in Lithuania.

Key words: Types, ornamental patterns, cemetery planting, Šiauliai.

1. Introduction

A cemetery as an eternal resting place of the dead is a rare object of research in Lithuania. The British, for example have a periodical publication “Mortality”, devoted to this subject. Many cemeteries located near major towns lack burial space, therefore it is likely that cremation and burial in urns will become more popular in the future, and will reduce the area needed for this purpose and prevent from turning of suburbs into entire cemeteries [1]. This, in turn, will bring changes in burial site designing traditions, and contemporary cemeteries with characteristic features will become history.

The issue of cemetery planting is not yet sufficiently studied in Lithuania. As lifestyle of the whole country changes, so do the cultural features and traditions of cemetery planting. New ways of decorating graveyards using plants appear, the choice of plants rapidly increases the social attitude towards the decoration and maintenance of such public spots changes, besides the life of plants is very much time-dependent. Cemetery planting has become a very dynamic phenomenon which is characterised by national peculiarities as well as significantly expanding cosmopolite features. Characteristic and valuable present-day realities soon will probably irreversibly change and pass into the category of historical heritage. It is essential, therefore, to analyze and record the existing peculiarities of cemetery planting in Lithuania which reflect cultural level and traditions of modern society and are important for future generations. By presenting this sphere of planting tradition to other nations we can demonstrate how different, unique and special we are.

Cemetery is one of the sites where people could give way to their subjective comprehension of beauty. Plants also serve this purpose. Burial site surface planted with various plants makes up one of the parts of architeconics of the recorded object (burial site). Analysis of the cemetery planting revealed certain noticeable features that could be ascribed to the forms of folk art. These are various objects with implied specific
content or ornamented compositions. An ornament as an element of décor is used in its various structural places: gravestone engravings, fences, layout of path tiles, candlesticks, lamps, vase décor, forged metal insertions integrated in the monument, layout of plants [2].

The ornament is formed by one or several rhythmically repeating elements which are located at the surface of an object regarding the entirety. Often the ornament holds not just decorative meaning, but serves as a representation of the surrounding world. In days of old the ornament, which originated from specific realistic stylized images, was used to communicate certain information by means of signs. The ornament could also have the already forgotten symbolic meaning.

By the methods of observation and analysis the graves with specific planting design, particular planting structure, peculiarities of plant arrangement or their composition were distinguished. The results obtained in both cemeteries and considering the periods of their formation were compared.

In 2007 such research results were:

- Plant range, popularity, colours, number of species and plant arrangement differ and largely depend on the grave formation period.
- The most popular genera were found to be: *Tagetes*, *Hosta*, *Petunia*, *Thuja*, *Begonia*, *Buxus*, *Sempervivum*, *Impatiens*, etc.
- The top tens of the most popular plants are different in the burial sites differing in the age; however, some plants are used irrespectively on the period of their formation.
- The diversity of plants planted in burial sites includes 64 major genera. The greatest plant diversity was identified in the moderately old burial sites.
- In terms of plant range, most of the plants are perennial.
- In the new burial sites the most popular are annual plants, and in old ones perennial plants. The latter are 2.5 times more frequent.
- The dominating colour of plant flowers in July is yellow.
- In the burial sites of various ages the plants of variegated flowers dominate equally. Single-colour burial sites are rare.
- The number of plant species per burial site varies from one to five. The most frequent is the use of two to three species.
- Characteristic plant arrangement is in the corners of a burial site and symmetric.
- Analysis of grave planting revealed several features specific to a large part of burial grounds and pointed out the newly emerging characteristics [3]:
  - Burial ground planting is most often unrelated to the material, shape, and colour of the monument, i.e., they are not matched. This suggests that landscape designers are not involved in the burial grounds’ planting process.
  - Specific shapes - hearts, crosses, suns formed from plants are increasingly used. There may be two crosses on one burial ground intended for each of the buried persons. These specific shapes are most often planted with *Saxifraga* L., *Leontopodium* R. Br. ex Cass., *Sempervivum* L., *Sagina* L., *Lobelia erinus* L.
  - Conifers are often planted beyond the borders of a burial ground and form a green background for the monument. The popularity of individual ornamentally trimmed conifers is increasing.
  - Plants that used to be considered as indoor pot plants (*Pelargonium* L’Hér., *Coleus* Lour., *Zantedeschia* Spreng., *Kalanchoe* Adans., *Sedum* L.) are increasingly used for grave planting. This was not characteristic of the period 15-20 years ago.
  - In terms of colours, on the background of black earth or black monument, the plants that are difficult to discern are those with red or dark leaves: *Impatiens hawkeri* Bull., *Heuchera micrantha* Lindley, *Lobelia erinus*, *Ajuga* L., *Perilla frutescens* L. Britton and others. Consequently, a better result is achieved when plants with light leaves are planted alongside the other light-leafed plants that make a contrast to dark-leafed plants, for example: *Senecio bicolor* (Willd.) Tod., *Antennaria*
Particular Ways of Planting in Cemeteries of Šiauliai City (Lithuania)

Gaertn., *Leontopodium*, *Saxifraga*, *Sedum* and others.

- Mottled-leafed plants (*Euonimus fortunei*: Emerald’n Gold, Aureomarginata, Argenteovariegata, *Ajuga*, *Juniperus sabina* Variegata and others), look nice on a burial ground only when they grow alone or at a certain distance from other plants, since leaf or needle glimmer creates the sense of disorder or excessive playfulness.

- A new trend in burial ground decoration is emerging. It is stone background made by placing stones under a plant. The trend has been directly transferred from garden design field.

- A characteristic grave decoration shape is rhomb. It is used not only for planting but also for laying tiles. There may be from one to four of such rhombi on a burial ground. It is likely that this shape comes from the primitive Lithuanian ornament.

- New varieties of short-growing conifers and drooping saplings imported from west Europe have been started to be used. Currently the number of such plants is not high since specialists find it difficult to choose them for a burial ground so that they do not obscure monument inscription and do not spread too much.

- Ornaments covered by plants have emerged in grave planting. This phenomenon is becoming increasingly popular. The ornaments represent flower, leaf or geometric motif. The most common short-growing plants used for this purpose are: *Armeria* Willd., *Hutchinsia* R. Br. *Iberis* L., *Leontopodium*, *Sagina subulata* (Sw.) C. Presl, *Sempervivum*, *Sedum*, *Festuca pellens* Host ir *Festuca scoparia* Hook. f..

In 2008, researches in Ref. [4] got these results:

Despite the extremely great diversity of burial grounds’ planting, there were distinguished four types of planting and twenty four standard planting layouts. Practically any of the burial grounds falls within one of these schemes. The most popular planting types were found to be longitudinal and central. Characteristic as well as newly emerged burial ground planting features were distinguished and only after a longer period it will be possible to state whether they will become well-established or will be abandoned.

The study suggests that the use of various burial ground covers is becoming increasingly popular. They tend to substitute plants because they require less management. Covers of non-plant origin are more characteristic of the new burial grounds. In summary, we can maintain that covers of non-plant origin are a rare phenomenon compared with plant covers.

The mean of burial grounds with differently planted surfaces (larger part, moderately planted, little planted) makes up from 29% to 39%, which does not differ significantly and still does not clearly express any valid trend.

Burial ground planting is most often unrelated to the material, shape, and colour of the monument. This indicates that burial planting is guided by individual amateurish understanding.

The researches about ratio of ornaments which regard to the layout of the structural parts and frequency of ornaments in burial sites in different periods of formation were described in author’s article *Plant ornaments in Šiauliai cemetery* [4].

The aim of 2010 research was to ascertain and distinguish graves characterised by specific planting features associated with plants, their layouts on graves.

Tasks of the research were:

(a) to determine and formulate criteria for planting of ornamented graves;

(b) to identify plants and determine the frequency of their application on ornamented graves;

(c) to distinguish specific objects depicted in planting layouts;

(d) to determine the frequency of symbolic plants application in planting of particular graves;

(e) to explore symbolic meanings of plant ornamentation.

Object of the research was plants on graves.

Analysis of various literature references allowed distinguishing most frequently used ornamental patterns: cross, sun, moon, triangle, rhombus,
mountains, fish, heart, tree. Majority of the authors [5-9] similarly interpret symbolic meaning of these patterns:

A cross—magical and religious symbol. It appeared in primitive society as a symbol of fire. It also symbolizes four elements, four parts of the world. In pre-Christian times it was used by Celts and pagans. In Christianity it is a symbol of Jesus Christ crucifixion and resurrection.

The sun—primary meaning—sky fire, flash, in primitive society the sun was worshiped as deity, producer of live. The sun symbolizes fire, perfection, eternity. Ornaments of grass-snake and snake are closely connected with the cult of the sun.

The moon—flash of the night, dissipating darkness. The symbol is worshiped since the Stone Age. The moon is associated with the world of the dead in Lithuanian patriarch period; it is considered the patron of the dead.

A triangle—one of the most sophisticated symbols. It symbolizes tree dimensions if the universe (God’s world, natural world, human world). Long ago the triangle symbolized father, mother and child. It is the symbol of birth, life and death.

A rhombus—this is the symbol of activity, with many meanings: fire, earth, sun, day, wreath, knot, and ring.

Mountains—cosmic center. They separate the main spheres, i.e., sky and earth; they also symbolize ascension and descending. In folklore it is a symbol of a cemetery.

A fish—associated with water, element of life. It is the symbol of fertility and death. Fish is one of the oldest secret symbols of Jesus Christ.

A heart—symbol of love, joy, sorrow, and mercy.

A tree—the cult of a tree exists worldwide during all times. It symbolizes pillar of the world. A tree shows the connection between underground space and cosmos, link between the sky, earth and underground, bond between the live and the dead as well as between the past, presence and future.

All these symbols could be found in grave planting layouts. Since burial sites’ planting is done by people who are not professional artists, ornamental planting can be considered as another folk art expression form.

2. Materials and Methods

Presently there are two functioning cemeteries in Šiauliai city (Lithuania): K. Donelaitis and Ginkūnai. K. Donelaitis cemetery was established in 1960 and now comprises about 5,000 graves. The allotted territory is almost completely used (9.74 ha). Ginkūnai cemetery is being used since 1972 and it is also almost full (34.36 ha). The research was performed in summer of 2007, 2009, and 2010. 2,000 graves in K. Donelaitis cemetery and 20,000 graves in Ginkūnai cemetery were analyzed (in total 22,000 graves).

Research methods were: analysis, plant identification, description of plant arrangement, photography, colleague discussions.

3. Results and Discussion

Analysis of the research results shows that two options form the peculiarities of grave planting: depiction of specific objects and ornamentation. Wider explanations should be given regarding these two groups. Real things and visual symbols are ascribed to specific objects. Visualization employing plants make them already stylized because exclusive shapes and textures form due to the structure of plant material. In cemeteries of Šiauliai city the following planting layouts were distinguished: crosses, suns, moons, hearts, trees, mountains, fish and geometric forms (most frequently rhombi and triangles). According to display of symbolic objects, the graves could be divided into three groups:

(1) the entire object is depicted;
(2) part of the object is depicted (a half or quarter of a sun, half-moon, etc.);
(3) several symbolic objects are depicted.

During this research the graves planted with symbolic objects and ornaments were distinguished. This phenomenon becomes more and more frequent.
Cross shaped plantings are usually planted single or two on one grave (Fig. 1). They all are asymmetrically composed. Shape of a cross is frequently planted using the same plant species, but sometimes it is contoured using other plants or non-plant materials. The choice of the layout is most probably determined by the meaning and significance of the symbol.

Display of the sun is one of the most frequently used. The whole sun, half of it or just a quarter could be depicted (Figs. 2 and 3). Usually the whole sun and its rays are planted using the same plants. The symbol of light kind of divides the two worlds, somewhat cheering up the appearance of a grave.

The heart shaped planting is infrequent. It could be a large (cover up to half of a grave) or a small one. In all cases only contour depicts the heart (Fig. 4). The heart symbol expresses sorrow, affection, love, faithfulness.

The tree shaped layout is probably the most problematic considering its appearance because it is mostly formed of branch system and, therefore, much detached from the realistic image of a tree (Fig. 5). Such view evokes associations related with life; the broken branch is associated with the end of life.

Image of a moon is also rather rare in grave planting. It is usually linked with covering of the remaining grave surface, so it is not very spectacular and rather difficult to distinguish (Fig. 6). The moon is the symbol of night,
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darkness. Solitary image of a sickle strongly associates with the Muslim symbol that is why the moon is depicted as half of a circle.

Image of mountains is displayed rather realistically. They are rather hills than real mountains (Fig. 7), symbolizing the life journey, ascensions and falls. Even, rounded tops of these hills are neatly waving in undersize plants.

The image of a fish was a real surprise for the researchers. The layout is formed of fish images as if swimming in one direction or in opposite directions (Fig. 8). The layout is formed as “yin” and “yang” images from oriental culture. Regular layout shapes, playful color patterns.

The most popular is depiction of geometric forms: rhombus (Fig. 9) and triangle (Fig. 10). We think that these two shapes were used for grave planting since very old times. Rhombi are either only contoured or fully planted inside, usually by plants of different genera. The rhombi are formed of plants or other materials. The rhombi are attractive due to their order, simple shape. The size of these rhombi varies from 30 cm to the size covering the whole grave. Right-angled and isosceles triangle shapes are used in cemetery planting. Their shapes are easily formed on graves; the number of triangles varies from one to three per grave.
Ornamental planting of graves is a rare phenomenon. It could be stated that the appearance of this phenomenon is not really recorded. Ornamented graves make only 0.18% [3] of the graves in Šiauliai city. The recorded ornaments were grouped into:

1. Geometric: straight lines, curved lines, geometric forms;
2. Floral: flowers, branches, seeds;
3. Combined.

Geometric ornaments containing straight lines are characterised by different numbers and directions of the lines (Figs. 11-12).

Most frequent pattern is the repetitive diagonal lines or lines perpendicular to the grave contour. Rhythmically occurring lines formed of different plants, e.g., Saxifraga, Petunia and Armeriamaritima, Saxifraga and Armeriamaritima. Probably, particularly complex are line ornaments formed of fluent junctions (Fig. 13). These are much more difficult to compose and plant because much precision is needed. We could not tell if the owners designed them themselves or copied from somewhere. Usually such patterns cover most surface of the grave, but elements located only at the corners of graves also occur. Ornaments of curved lines are almost always planted employing Sempervivum. Ornaments of geometric forms are most frequently formed of rectangles and rhombi of various sizes (Fig. 14). Geometric forms could be entirely filled with plants or have just a planted contour.

Floral ornaments are formed of the repetitive elements of depicted plants: flowers (Fig. 15), branches (Fig. 16), seeds (Fig. 17). Flowers are usually presented with stalks. Different plants are used for depicting of flower and leaves, e.g., Petunia (flower) and Saginasubulata (stalk with leaves) or Pelargonium × hortorum (flower) and Festucaglauca with Saxifraga.

Fig. 11 Perpendicular lines.

Fig. 12 Repetition of diagonal lines.

Fig. 13 Ornament of fluent junctions.

Fig. 14 Ornament formed of rectangles.
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(stalk with leaves). Branches are usually depicted in checkerboard pattern. Their patterns are planted using Armeriamaritima or Sempervivum plants. Just a single example of seed presentation was recorded.

Maybe people do not notice their beautiful shapes and meanings in grave planting. Combined ornaments are not typical either (Fig. 18). Usually straight and curved lines interchange.

We think that majority of the ornaments are made with the single purpose of decoration, and do not associate with symbolic meanings. Registry of all plants

Fig. 15 Flower ornament.

Fig. 16 Branch image.

Fig. 17 Seed image.

Fig. 18 Combined ornament.

Table 1 Plants used for formation of specific object or ornament layouts

<table>
<thead>
<tr>
<th>No.</th>
<th>Perennial plants</th>
<th>Number of graves</th>
<th>No.</th>
<th>Annual plants</th>
<th>Number of graves</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Sagina subulata</em> (Sw.) C. Presl.</td>
<td>22</td>
<td>1.</td>
<td><em>Begonia semperflorens</em> L.</td>
<td>12</td>
</tr>
<tr>
<td>2.</td>
<td><em>Sempervivum</em> L.</td>
<td>18</td>
<td>2.</td>
<td><em>Impatiens walleriana</em> L.</td>
<td>10</td>
</tr>
<tr>
<td>5.</td>
<td><em>Antennariadioica</em> (L.) Gaertn.</td>
<td>6</td>
<td>5.</td>
<td><em>Petunia Juss.</em></td>
<td>8</td>
</tr>
<tr>
<td>8.</td>
<td><em>Aubrieta</em> Adans.</td>
<td>2</td>
<td>8.</td>
<td><em>Dianthus</em> L.</td>
<td>2</td>
</tr>
<tr>
<td>11.</td>
<td><em>Campanula carpatica</em> Jacq.</td>
<td>1</td>
<td>11.</td>
<td><em>Gazaniarigens</em> Gaertn.</td>
<td>1</td>
</tr>
<tr>
<td>12.</td>
<td><em>Primula</em> L.</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
used for various layouts of cemetery planting (Table 1) revealed that both annual and perennial plants are used. They all are characterised by small height (2-15 cm), moderate spread, a rather well defined shape of a plant.

These characteristics make possible to form rather complicated and small shapes where the plant color is of no particular importance. The ratio between the diversity of perennial and annual plants is very similar, i.e. 12:11. The most popular perennials are *Sagina*, *Sempervivum*, *Sedum* plants; most popular annuals are *Begonia*, *Impatiens Walleriana*. Symbolic objects could be depicted in three ways: (1) entire object; (2) part of an object (half or quarter of the sun, half-moon, etc.); and (3) several repetitive objects.

During this research the graves decorated with planted symbolic objects and ornaments were distinguished. This phenomenon becomes more and more popular in cemeteries of Šiauliai city.

4. Conclusions

Application of plants for depiction of specific objects and ornaments adds to the particularity of cemetery planting.

As cemetery planting is usually performed not by professional landscape designers but by amateurs, the plants are probably randomly chosen.

Geometric ornaments are characterised by three structural elements: straight lines, curved lines and geometric forms. Floral ornaments are characterised by presentation of plant structural parts: flowers, branches and seeds.

Presentation of specific objects by plants is frequently depicted by their contours rather than patterns entirely filled with plants.

Nearly equal numbers of annual and perennial plant genera are used for grave planting (in total 23 genera).

Most popular plants in ornamental planting are *Sagina subulata*, *Sempervivum*, *Begonia semperflorens*, *Sedum acre*, *Impatiens walleriana*, *Begonia tuberhybrida*, *Tagetes patula*, *Petunia*.

References

Influence of Trap Construction on Mosquito Capture

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Abstract: During 2009 and 2010, 23 night-time mosquito captures were made at Kančí obora in south-eastern Moravia, Czech Republic. It was used in the Centers for Disease Control and Prevention (CDCP) miniature light traps with CO₂ (dry ice) and baited lard-can traps in which sentinel animals were replaced with a container filled with CO₂ (dry ice). In the observed period, a total of 31,882 female mosquitoes were captured by CDC miniature light traps with CO₂. Lard-can traps baited with CO₂ captured 995 females under the same conditions, which is just 3.12% of the quantity from the CDC traps. At the same time, there were significant differences in the proportional captures of various species. Compared to CDC miniature light traps, baited lard-can traps much more often captured Aedes cinereus (16.58% of total versus 1.93% in CDC traps), Culex modestus (15.48% versus 4.62%), and Ae. rossicus (6.13% versus 2.67%). On the other hand, capture of female Ae. vexans was proportionally much lower (15.38% versus 36.41%). Capture of Cx. pipiens was more or less the same 14.77% (miniature light traps) and 15.76% (baited lard-can traps). The occurrence of the calamity species Ae. sticticus was proportionally very high in both trap types (30.05% in lard-can traps baited with CO₂, 33.58% in CDC miniature light traps). The findings prove that a trap’s design itself significantly affects not only the overall capture of mosquitoes but also the proportional representation of individual species.

Key words: CDC miniature light traps, baited lard-can traps, Aedes cinereus, Aedes vexans, Aedes sticticus, Culex modestus, Culex pipiens.

1. Introduction

Research on mosquitoes often depends upon capturing the females. For this purpose, a number of methods have been developed and many ingenious devices created. Mosquitoes are baited using various animals or chemical compounds (most frequently CO₂). Common devices include in particular CDC miniature light traps with CO₂ [1-5], but also traps baited with live animals. Attractants used have included live ducks [6], pigeons [5, 7], chickens [8, 9], starlings (Sturnus vulgaris) [7], and horses [2, 6]. Sometimes, even mosquitoes attacking humans are collected [2, 10].

Numerous studies have compared the effectiveness of different trap types and attractants [11-14]. A comparison of CDC traps using various attractants (CO₂, octenol, light) and their combinations were made by Becker et al. [15].

In addition to the type of trap and attractant used, another important factor is the height at which the trap is situated [5, 16, 17]. The present work aims to verify how mosquito captures and their species representation are influenced by the structure of the baited lard-can traps themselves.

2. Material and Methods

2.1 Sites

The Kančí obora site (48°46’N, 16°52’E, 157 m a.s.l.) is located in south-eastern Moravia, Czech Republic (Fig. 1) and is comprised primarily of floodplain forest. The dominating trees are Quercus robur L., Fraxinus angustifolia Vahl, Populus spp.,
Influence of Trap Construction on Mosquito Capture

*Tilia cordata* Mill, and *Carpinus betulus* L.. The floodplain forest is often flooded with water from the Dyje River. The traps were located approximately 500 m from the town of Bréclav.

2.2 Meteorological Data

South-eastern Moravia is characterized by a relatively warm and dry climate. Average daily temperature is 9.3 °C and average total annual precipitation is 490 mm.

The studied period, 2009 and 2010, had above-average precipitation (Fig. 2).

During January-October (end of capturing) 2009, precipitation totaled 594.1 mm (113.8% of the norm). In 2010, this figure was 681.7 mm (161.5% of norm) (data from Czech Hydrometeorological Institute’s Kobyli station, 19 km north of the site).

In 2009, however, only March, June and July had above-average precipitation, which was reflected in a high incidence of mosquitoes especially in summer. In 2010, high precipitation was recorded for the majority of the observed period and the overall mosquitoes incidence was also distinctly higher.

Fig. 1  Map of study sites in the Czech Republic.

Fig. 2  Monthly sum of precipitation (mm × 10) and Mean monthly air temperature (°C) in the study area, compared with the long-term average (Kobyli; data from Czech Hydrometeorological Institute in Brno).
2.3 Trapping Method

We used two types of traps for trapping female mosquitoes:
(a) CDC miniature light traps with CO$_2$ (BioQuip Products, Inc., Rancho Dominiquez, CA, USA.), supplemented with 1.5 kg of dry ice.
(b) Baited lard-can traps [7] in which the sentinel animal was replaced by a container with 1.5 kg of dry ice (Fig. 3). The container was made from polystyrene foam with dimensions 260 × 170 mm. Both smaller sides were provided with three circular apertures 0.6 mm in diameter. Two polystyrene barriers 110 mm high were inserted into the container. By inserting the barriers the appropriate discharge of CO$_2$ was achieved.

The traps were installed 1 m high and approximately 25 m from one another. Lard-can traps without bait were used as control. The exposure was throughout the night from 16:00 to 8:00 Central European Summer Time. Collections were made from the beginning of April until the end of October and a total of 23 collections were made.

2.4 Identification

Keys by Kramář [18] and Becker [19] were used.

2.5 Statistical Analysis

The relative abundance of each species was calculated separately for each monitored period. The following scale of dominance was used: more than 10% of the total number of culicidae captured per studied period was regarded as eudominant (ED), 5-10% as dominant (D), 2-5% as subdominant (SD), 1-2% as recedent (R), and less than 1% as subrecedent (SR). The index of dominance (C), Shannon-Weaver diversity index (H’), and equitability index (E) were monitored for each period.

3. Results

The capture of female mosquitoes in both types of traps displayed significant differences in both quantity as well as qualitative representation. CDC miniature light traps with CO$_2$ captured 31,882 females through the season. At the same time, lard-can traps with CO$_2$ captured 995 females, or just 3.12% of the number captured by CDC traps. Only 19 individuals flew into the empty lard-can traps (Table 1). In samples collected from CDC miniature light traps with CO$_2$, the calamity species *Aedes vexans* (Meigen) (36.41% of total) and *Ae. sticticus* (Meigen) (33.58%) significantly dominated, followed by *Culex pipiens* Linnaeus (15.76%) and *Cx. modestus* Ficalbi (4.62 %) (Fig. 4). *Aedes sticticus* was also very abundant in lard-can traps with CO$_2$ (30.05%). Other common species in this type of trap were *Cx. modestus* (15.48%) and *Cx. pippins* (14.77%). Compared to CDC traps, there was a relatively low occurrence of *Ae. vexans*, which represented just 15.38% of the total here. This type of trap, however, seemed to be attractive for the species *Ae. cinereus* Meigen (16.58% of the total versus 1.93% in CDC traps), and a little less so for *Ae. rossicus* Dolbeskin, Gorickaja and Mitrofanova (6.13% versus 2.67%) (Fig. 5). The numbers of females of individual species captured in the lard-can traps with CO$_2$ compared to the capture by CDC miniature light traps with CO$_2$ (expressed in %) and the representation of some of the mosquito species in the different trap types are displayed in Figs. 6 and 7.

Fig. 3  Lard-can traps baited with CO$_2$. 
Table 1  List of species collected on the locality Kančí obora, including number of individuals (No), relative abundance (%), and classification of dominance (CD) (eudominant–ED; dominant–D; subdominant–SD; recedent–R; subrecedent–SR), ED and D are accentuated by bold face.

<table>
<thead>
<tr>
<th>Species</th>
<th>CDC miniature traps</th>
<th>lard-can traps baited with CO₂</th>
<th>lard-can traps baited without CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
<td>CD</td>
</tr>
<tr>
<td>An. maculipennis s. L.</td>
<td>99</td>
<td>0.31</td>
<td>SR</td>
</tr>
<tr>
<td>An claviger</td>
<td>22</td>
<td>0.07</td>
<td>SR</td>
</tr>
<tr>
<td>An. plumbeus</td>
<td>22</td>
<td>0.07</td>
<td>SR</td>
</tr>
<tr>
<td>Ae. cantans s. L.</td>
<td>1,078</td>
<td>3.38</td>
<td>SD</td>
</tr>
<tr>
<td>Ae. caspius</td>
<td>1</td>
<td>0.00</td>
<td>SR</td>
</tr>
<tr>
<td>Ae. cataphylla</td>
<td>116</td>
<td>0.36</td>
<td>SR</td>
</tr>
<tr>
<td>Ae. cinereus</td>
<td>616</td>
<td>1.93</td>
<td>R</td>
</tr>
<tr>
<td>Ae. excrucians</td>
<td>27</td>
<td>0.08</td>
<td>SR</td>
</tr>
<tr>
<td>Ae. geniculatus</td>
<td>9</td>
<td>0.03</td>
<td>SR</td>
</tr>
<tr>
<td>Ae. rossicus</td>
<td>851</td>
<td>2.67</td>
<td>SD</td>
</tr>
<tr>
<td>Ae. sticticus</td>
<td>10,705</td>
<td>33.58</td>
<td>ED</td>
</tr>
<tr>
<td>Ae. vexans</td>
<td>11,607</td>
<td>36.41</td>
<td>ED</td>
</tr>
<tr>
<td>Cx. modestus</td>
<td>1,472</td>
<td>4.62</td>
<td>SD</td>
</tr>
<tr>
<td>Cx. pipiens</td>
<td>5,024</td>
<td>15.76</td>
<td>ED</td>
</tr>
<tr>
<td>Cs. annulata</td>
<td>96</td>
<td>0.30</td>
<td>SR</td>
</tr>
<tr>
<td>Cq. richiardii</td>
<td>137</td>
<td>0.43</td>
<td>SR</td>
</tr>
<tr>
<td>Total specimens</td>
<td>31,882</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total species</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H’</td>
<td>1.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.56</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4  Representation of the individual mosquito species captured by CDC miniature light traps with CO₂.
Influence of Trap Construction on Mosquito Capture

Fig. 5  Representation of the individual mosquito species captured by lard-can traps baited with CO₂.

Fig. 6  Representation of the females of individual mosquito species captured by lard-can traps baited with CO₂ in comparison to the numbers captured by CDC miniature light traps with CO₂.

Fig. 7  Percentage representation of selected mosquito species in different trap types.
A: lard-can traps baited with CO₂ (2009-2010); B: lard-can traps baited with a live pigeon (2007-2008); C: CDC miniature light traps with CO₂ (2009-2010); D: CDC miniature light traps with CO₂ (2007-2008).
4. Discussion

Hoel et al. [20] made a comparison of six traps from the viewpoint of capturing *Ae. albopictus*. These authors compared the following traps: mosquito magnet professional, fay-prince, CDC wilton, mosquito magnet-X, mosquito magnet liberty, and the standard CDC light trap. In addition to CO₂, the attractants L-lactic acid and octenol were used. Individual traps differed not only by total number of captured mosquitoes (in a range of 2,145-11,143 adults) but also by proportional representation of individual mosquito species. The monitored *Ae. albopictus* comprised 14.2% of the total number of captured adults (in individual types of traps ranging from 3.3% to 63.6%). The mosquito species represented in that work included no species occurring on the territory of the Czech Republic.

Research on species composition comparing the two trap types also had been conducted in south-eastern Moravia during 2007 and 2008 [5]. Female mosquitoes were captured at two sites nearby to one another: Nesyt (located 12 km from Kančí obora) and Soutok (about 15 km away) (Fig. 1). At that time, CDC miniature light traps with CO₂ and lard-can traps baited with a live pigeon had been used. During this research, 6,836 female mosquitoes were captured using three CDC miniature light traps hung at 1 m height. The most abundant species was *Ae. vexans* (72.95% of total). Another species with higher occurrence were *Cx. pipiens* (6.60%), *Ae. cantans* s.l. (*Ae. cantans* + *Ae. annulipes* Meigen) (5.82%) and *Ae. cinereus* 1.24%. Meanwhile, three lard-can traps baited with a live pigeon captured 213 females (3.06% of the number captured by CDC miniature light traps). *Cx. pipiens* comprised 93.42% of the total, *Ae. vexans* only two females in total, and *Ae. cinereus* was not represented here. The trap was clearly selective, with high dominance of the ornithophilous species *Cx. pipiens* compared to CDC miniature light traps with CO₂ (Fig. 7, Table 1).

In comparing the results of the two studies, it is evident that the numbers of mosquitoes captured by lard-can traps baited with CO₂ or with a live pigeon are distinctly lower compared to the numbers captured by CDC miniature light traps with CO₂. The two cases using lard-can traps are comparable to one another when their capture numbers are expressed as percentages of the corresponding CDC traps capture (3.12% with CO₂ and 3.06% with a pigeon). The spectrum of species captured by baited lard-can traps is markedly influenced by the species of sentinel animal used, but the trap structure itself was partially selective (Fig. 7). When using just CO₂ as the attractant, baited lard-can traps were preferred by the species *Ae. cinereus*, *Ae. rossicus* and *Cx. modestus*, while the findings of the most abundant species *Ae. vexans* were decisively and negatively influenced.

Differences in mosquito captures, both quantitative and qualitative, when using various types of traps have been established also by other authors [11, 12]. This points to the need to take into account this fact when interpreting results and emphasizes the importance of correct trap choice for a specific situation.

5. Conclusion

The results of this work show that the type of trap and its design significantly influence not only overall mosquito capture but also the proportional representation of the individual species. When planning research, therefore, due attention must be given to the choice of traps.

Acknowledgments

The research was supported by Grant No. 2B08003 from the Ministry of Education of the Czech Republic and Project Grant No. Z50070508 of the Academy of Sciences of the Czech Republic.

References

Influence of Trap Construction on Mosquito Capture


Spatial and Temporal Distribution of Oncaeidae in Chabahar Bay, Gulf of Oman

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Abstract: The spatial and temporal distribution patterns of the zooplankton of Chabahar Bay, Gulf of Oman were investigated. Zooplankton sampling was collected twice a season at five stations in Chabahar Bay. Sampling was done during July-August 2007 (SW-monsoon), October-November 2007 (post-monsoon), January-February 2008 (NE-monsoon), and March-May 2008 (pre-monsoon). Five stations were investigated throughout Chabahar Bay. Four species of Oncaeidae (Oncaea media, Oncaea minuta, Oncaea venusta and Oncaea clevei) were identified. The abundance of Oncaea media was maximum in the post-monsoon (> 700 ind.·m⁻³) and disappeared in pre-monsoon while Oncaea minuta was maximum in post-monsoon (> 130 ind.·m⁻³) and disappeared in NE-monsoon and pre-monsoon. Oncaea venusta showed the highest abundance in post-monsoon (> 370 ind.·m⁻³) and the lowest in pre-monsoon (< 55 ind.·m⁻³). The highest abundance of Oncaea clevei was in post-monsoon (< 240 ind.·m⁻³) and lowest in NE-monsoon. Overall, the highest abundance of Oncaeidae was observed in post-monsoon. The results showed that depth was the most important factor controlling abundance of the Oncaeidae. Spatially, the highest abundance of Oncaeidae species was found in off shore stations. Four species of this family showed positive correlation with depth. Also, O. venusta showed negative correlation with salinity that showed this species prefers low saline water.

Key words: Abundance, Oncaeidae, monsoon, depth, salinity, Chabahar Bay.

1. Introduction

Chabahar Bay is a small semi-enclosed and sub-tropical bay on the southeastern coasts of Iran (25°17'45"N - 60°37'45"E). The bay surface area is 290 km² with 14 km wide is located between Chabahar and Konarak (Fig. 1). The average depth of the bay is 12 m (ranges from 8-22 m). This bay is connected to the Indian Ocean by the Gulf of Oman. Therefore, the effect of Indian monsoonal winds on this area is remarkable.

The year is divided into periods of northeast (NE) monsoon and southwest (SW) monsoon and its following inter-monsoon periods (post-south west (post-monsoon) monsoon and pre-southwest monsoon (pre-monsoon) [1, 2]. The bay is one of the five major ports in the Arabian Sea and Gulf of Oman. However, very little published informations are available on the bay [1]. Copepods are the representative taxa of pelagic mesozooplankton and are both highly diverse and abundant [3]. The importance of zooplankton and copepod in marine pelagic food webs as food for larval fish has been shown [4, 5]. Nonetheless there are some information based on copepoda in Mussa creeks [6, 7] and based on total zooplankton in Ropme Sea [8], in Arabian Sea [9, 10], in waters of Pakistan [6, 11] in Arabian Sea and Indian Ocean [12-16]. The present study is one of the few in the region to examine the abundance and diversity of Oncaeidae (temporal-spatial variation) and to determine the effect of environmental factors on abundance.

2. Materials and Methods

Oncaeidae samples were collected from zooplankton
twice a season, during July-August 2007 (SW-monsoon), October-November 2007 (post-monsoon), January-February 2008 (NE-monsoon), and March-May 2008 (pre-monsoon). Five stations were investigated throughout Chabahar Bay. Two stations (St. 1 and 2) were located far from shore waters 22 m depth, another two stations were located near shore with 6 m depth (St. 3 and 5) and the final station (St. 4) was located in the middle of the bay with 12 m depth. Zooplankton was collected by using of 100 µm mesh nets equipped with a hydrobios flow meter.

Plankton samples fixed immediately in 4-5% formalin, buffered to a pH of 8 with sodium tetraborate (borax) [17]. Two-way ANOVA was used to assess the significant difference in abundance amongst periods and locations. The Pearson correlation and multivariate regression analyses were performed to determine the significance between environmental parameters and Oncaeidae abundance.

3. Results and Discussion

3.1 Environmental Parameters

The water temperature ranged from 23.10 ± 0.13 °C to 30.30 ± 0.45 °C in January and July; salinity ranged from 36.3 ± 0.03 Psu in May to 36.91 ± 0.14 Psu in October (Fig. 2). The average chlorophyll-a concentrations ranged from 0.56 ± 0.08 mg·m⁻³ in July to 2.00 ± 0.92 mg·m⁻³ in February.

Minimum and maximum values of dissolved water oxygen were from 5.42 ± 0.05 mL·L⁻¹ in May to 8.00 ± 0.61 mL·L⁻¹ in January.

3.2 Temporal and Spatial Variation

Mean seasonal abundance of *Oncaea* varied considerably. The abundance of *O. media* was maximum in the post-monsoon (725.30 ind·m⁻³) and
disappeared in pre-monsoon (Table 1). *O. venusta* showed the highest abundance in post-monsoon (> 350 ind·m⁻³) and lowest in pre-monsoon (< 60 ind·m⁻³). This species showed the highest abundance in Taiwan Strait [18]. The highest abundance of *O. clevei* was in post-monsoon (> 200 ind·m⁻³). The lowest abundance was observed in NE-monsoon.

*O. minuta* was found only in post-monsoon (134 ind·m⁻³) and SW-monsoon (> 8 ind·m⁻³). This species disappeared in other seasons.

There are some studies in Oncaeidae abundance in other tropical waters. *O. spp.* increased in dry season in Amazon estuary [19]. It was stated that *O. media* showed the highest abundance in summer in Taiwan Strait [18].

Spatially, *O. media* abundance ranged from 0 to 4,100.01 ind·m⁻³ in November in station 1. This species completely disappeared in March and May (Fig. 3). There was no significant difference (*P* > 0.05) between abundance of *O. venusta* between stations.

However, their abundance ranged from 0 to 477.81 ind·m⁻³. Maximum number of *O. clevei* was found at

**Table 1** Abundance of species of Oncaeidae in Chabahar Bay (ind·m⁻³).

<table>
<thead>
<tr>
<th>Variables</th>
<th>SW-monsoon</th>
<th>Post-monsoon</th>
<th>NE-monsoon</th>
<th>Pre-monsoon</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. media</em></td>
<td>121.65</td>
<td>725.30</td>
<td>581.56</td>
<td>-</td>
</tr>
<tr>
<td><em>O. venusta</em></td>
<td>140.60</td>
<td>372.07</td>
<td>126.84</td>
<td>51.94</td>
</tr>
<tr>
<td><em>O. clevei</em></td>
<td>83.54</td>
<td>234.84</td>
<td>32.00</td>
<td>77.34</td>
</tr>
<tr>
<td><em>O. minuta</em></td>
<td>8.81</td>
<td>134.00</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Fig. 3 Abundance of Oncaeidae in Chabahar Bay in stations.](image-url)
Table 2  Pearson correlation of environmental parameters and Oncaeidae abundance.

<table>
<thead>
<tr>
<th>Variables</th>
<th>DO (mL·L⁻¹)</th>
<th>Salinity (Psu)</th>
<th>Temperature (°C)</th>
<th>Chl-a (mg·m⁻³)</th>
<th>Depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. media</td>
<td>-0.05</td>
<td>-0.39</td>
<td>-0.32</td>
<td>0.05</td>
<td>0.55*</td>
</tr>
<tr>
<td>O. venusta</td>
<td>-0.11</td>
<td>-0.52*</td>
<td>-0.14</td>
<td>0.12</td>
<td>0.72**</td>
</tr>
<tr>
<td>O. clevei</td>
<td>-0.14</td>
<td>-0.23</td>
<td>0.00</td>
<td>-0.08</td>
<td>0.46*</td>
</tr>
<tr>
<td>O. minuta</td>
<td>-0.08</td>
<td>-0.35</td>
<td>0.60</td>
<td>0.09</td>
<td>0.48*</td>
</tr>
</tbody>
</table>

*: significant at 0.05 level; **: significant at 0.01 level.

station 1 (1043.30 ind·m⁻³) in November. O. minuta was observed in low abundance in the whole year. Maximum abundance was found in station 1 in October and disappeared in stations 3, 4 and 5 and in January, February, March and May. Actually, this species was not found during NE-monsoon and pre-monsoon.

The seasonal variation in abundance of copepod in Chabahar Bay is regulated by environmental parameters [20]. The positive association between depth and O. media, O. minuta, O. venusta and O. clevei abundance (P < 0.05) reveals that depth is the major factor controlling the abundance of Oncaeidae in Chabahar Bay (Table 2).

In some researches, Oncaeae have been shown to be distributed from epipelagic to bathypelagic zones of several oceanic regions [21, 22]. It was stated that Oncaeid constitute an important part of the community in Arabian Sea, dominating especially in the meso- and bathypelagic zones [23]. Oncaea was abundant in the upper epipelagic zone in December and October in the Oyashio region. Some of the species are most common epilanktonic species from Pakistan offshore waters [24]. However a significant correlation was not observed between abundance of Oncaea and salinity in estuary of Bilbao [25].

It was stated that copepod abundance and distributions are influenced by hydrographic conditions in tropical waters [26, 27]. However, chlorophyll-α, temperature and DO appears to play a minor role in influencing abundance and distribution patterns of Oncaea in Chabahar Bay. No significant relationship was found between those parameters and Oncaea abundance. O. venusta was only species that showed positive correlation with salinity.

A similar trend was observed for the some studies in Arabian Sea and Indian Ocean [12-14]. They showed that salinity plays a key role in copepod abundance in Arabian Sea and Indian Ocean. In an opposite trend there was not significant correlation between O. spp. and salinity in Basque estuaries [25].

There was no significant association between chlorophyll-α and abundance of Oncaea in Chabahar Bay.

It was observed positive relationship between chlorophyll-α and copepod abundance in Indian Ocean [6, 9, 15].

4. Conclusion

In conclusion, spatial and temporal variations in Oncaea throughout the Chabahar Bay can be related to variations in the environmental variability; and depth and salinity play a major role in determining spatial and temporal patterns of Oncaea distribution and abundance.

Acknowledgments

The authors would like to thank Dr. Irina Prusova for her kindly suggestions for the experiment.

References


Diversity and Distribution of Tunicata (Urochordata) in Tobago

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Abstract: The beautiful island of Tobago is the southernmost Caribbean island. The sister island of Trinidad belongs to the Republic of Trinidad and Tobago. Thirty-two species of tunicates were collected from Tobago from depths of 40 m or less and they were listed. Tunicates listed in this work were from collections made in 1956, 1991, 1993, 2002 and 2006 and although specimens were collected from the Atlantic Ocean side of the island and the Caribbean Sea side, all species turned out to be typical Caribbean species.

Key words: Tobago, tunicates, Caribbean sea, taxonomy.

1. Introduction

Tunicata, commonly referred to as Tunicates or sea squirts, are among the highest of the invertebrates because they constitute a subphylum of the phylum Chordata (animals with a notochord or backbone). This classification is justified because they exhibit a notochord and a dorsal hollow nerve cord in the tail (resembling a tadpole) during their larval stage that is used for swimming. In nearly all species of tunicates, once the ideal habitat is found and the tadpole settles, as the animal develops into adulthood, the notochord is reabsorbed and the nerve cord shrinks to a simple ganglion. The name “tunicate” comes from the outer covering called the tunic. They are filter feeders, feeding on tiny particles especially bacteria from the water. There are no known freshwater species of tunicates, all are marine. The known classes of tunicates are Ascidiacea, Thaliacea, Appendicularia and the deepwater Sorberacea is not included here.

Class Ascidiacea consists of sessile (attached to a substrate) tunicates. Both solitary and colonial forms occur. Colonies are formed through asexual budding. Characteristic of this class is the tunic, the branchial siphon which receives nutrient rich current and the atrial siphon for excretion. The anatomy of an individual or zooid consists of the oral siphon, branchial sac (pharynx), stomach, gonads, intestine, rectum, heart, atrial cavity and atrial siphon. Ascidians, as they are called, are classified by the position of the gonads, the structure of the branchial sac and in many cases the larvae. All ascidians have a ventral heart. The direction of the heartbeat is reversed periodically. Ascidians accumulate heavy metals such as vanadium or iron in the blood.

The three orders of class Ascidiacea are Aplousobranchia, Phlebobranchia and Stolidobranchia. The order Aplousobranchia consists of compound species, having the body divided into two or three parts or segments (thorax, abdomen and sometimes post-abdomen). Asexual reproduction or budding occurs in this order. The order Phlebobranchia consists of compound and solitary species. Individuals of this order have a branchial sac without folds, a system of internal longitudinal vessels is normally present in the
Diversity and Distribution of Tunicata (Urochordata) in Tobago

branchial sac. The order Stolidobranchia is the most highly specialized order, individuals are never divided into a thorax and abdomen [1]. Some species of Stolidobranchia have a branchial sac that has folds, and some species have tentacles that are branched; some individuals have a hepatic gland or liver and some has a kidney.

Class Thaliacea: these are free swimming pelagic tunicates. Thaliaceans are often considered a nuisance among zooplankton biologists, because they sometimes clog their nets in large numbers preventing the capture of other animals. The tunic is transparent. The muscles of the body wall form circular bands which contract, eject water and produce locomotion.

Class Appendicularia: these are also free swimming pelagic tunicates with a trunk and muscular tail supported by a notochord usually several times longer than the trunk. Most species are only a few millimeters long (including the tail), but some may be up to 8-9 cm. They do not have a typical tunic, just a mucus-like covering.

This work follows the classification of orders based on the structure of the branchial sac [2-3]. There are papers that classify the tunicate orders listed in this paper as suborders under two orders Enterogona and Pleurogona based on larval development and form [4-6]. Orders Aplousobranchia and Phlebobranchia are suborders of Enterogona and order Stolidobranchia is a suborder of Pleurogona.

Tobago lies just north of the mainland of Venezuela in South America. The island is among the Caribbean islands that have one side facing the Caribbean Sea and the other side facing out toward the Atlantic Ocean (Fig. 1), but along with Trinidad, location wise, it is practically part of Venezuela. The first published work including tunicates from Tobago was in 1976 [7], which included four species of tunicates as part of a study of the fauna from the Buccoo Coral Reef and Bon Accord Lagoon area. There have been a number of publications listing shallow water tunicates from the Caribbean [8-24]. However, this account is the first on the distribution of tunicates of Tobago.

Fig. 1  Map of Tobago showing the sites where the tunicates were collected.
2. Materials and Methods

In 1956, Dr. Waldo Schmitt, a curator of the Museum of Natural History at the time, collected tunicates among other invertebrates from Tobago. His collections were part of the Smithsonian-Bredin Expedition that involved the collecting of fauna from many of the Caribbean islands, including Tobago. The species that Dr. Schmitt collected were identified many years later and they are included in this work. The list also includes species from observations made by J.S. Kenny, who in 1976 did a study of Tobago, from The University of the West Indies in Trinidad.

From 1991 to 2006, under the direction of Dave Hardy of the National Oceanic and Atmospheric Administration (NOAA), four collecting trips were made in Tobago and specimens were collected at depths under 40 m by snorkeling, wading and diving.

All specimens were relaxed with magnesium chloride for several hours, fixed in 10% formalin and transferred to 70% alcohol for storage at the Museum of Natural History. The pelagic species was kept in 10% formalin.

3. Results

Most of the species collected were found attached to submerged roots of red mangrove trees, others were found attached to coral, rock or mollusk shells. Tables 1 and 2 show the following species distribution and occurrence:

### Table 1 Occurrence of Tunicate species in Tobago.

<table>
<thead>
<tr>
<th>Species</th>
<th>Locations</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>A</td>
</tr>
<tr>
<td><strong>Family Polyclinidae</strong></td>
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</tr>
<tr>
<td>Aplidiopsis stellatus</td>
<td></td>
</tr>
<tr>
<td>Aplidium constellatum</td>
<td>X</td>
</tr>
<tr>
<td>Aplidium exile</td>
<td></td>
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<tr>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Didemnum duplicatum</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
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<tr>
<td>Trididemnum orbiculatum</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
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<tr>
<td>Clavelina oblonga</td>
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<tr>
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<td>Eudistoma clarum</td>
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<tr>
<td>Cystodytes dellechiaiei</td>
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</tr>
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<tr>
<td>Ascidia curvata</td>
<td></td>
</tr>
<tr>
<td>Ascidia interrupta</td>
<td></td>
</tr>
<tr>
<td>Phallasia nigra</td>
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<td><strong>Family Perophorididae</strong></td>
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<td>Perophora formosana</td>
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## Table 1 continued

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<tr>
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<td>Styela sp.</td>
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<table>
<thead>
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<tbody>
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<tr>
<td>Symplegma viride</td>
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</table>

<table>
<thead>
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<th>Locations</th>
</tr>
</thead>
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<td>Botryllodes nigrum</td>
<td>E</td>
</tr>
<tr>
<td>Botryllus planus</td>
<td>F</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Family Pyuridae</th>
<th>Locations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herdmania momus</td>
<td>X</td>
</tr>
<tr>
<td>Microcosmus anchylodeirus</td>
<td>X X X X X</td>
</tr>
<tr>
<td>Microcosmus exasperatus</td>
<td>X</td>
</tr>
<tr>
<td>Pyura vittata</td>
<td>X X</td>
</tr>
<tr>
<td>Salpa fusiformis</td>
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</tr>
</tbody>
</table>

Note: Codes for the different localities: A: Lover’s beach, Man-O-War bay; B: Goat island in Batteaux bay; C: Bon accord lagoon; D: Buccoo coral reef in Buccoo bay; E: Pirates cove in Man-O-War bay; F: Angel reef in Batteaux bay; G: Petit trou lagoon; H: Pigeon point; I: St. Giles and Melville islands; J: Man-O-War bay; K: Booby point of Booby bay; L: Brothers rock in Bloody bay; M: Corvo point; N: North point; O: Marble island in Man-O-War bay.

## Table 2  List of Tunicate species from Tobago island, in alphabetical order including US National Museum (USNM) catalog numbers and depths.

<table>
<thead>
<tr>
<th>Name</th>
<th>USNM #</th>
<th>Depth (in meters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aplidopsis stellatus</td>
<td>1014951</td>
<td>0-1 m</td>
</tr>
<tr>
<td>(Monniot &amp; Monniot, 1984)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aplidium constellatum (Verrill, 1871)</td>
<td>1014946</td>
<td>0-1 m</td>
</tr>
<tr>
<td>Aplidium constellatum (Verrill, 1871)</td>
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<td>1.5-2 m</td>
</tr>
<tr>
<td>Aplidium exile(Van Name, 1902)</td>
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<td>3-4 m</td>
</tr>
<tr>
<td>Ascidia curvata (Traustedt, 1882)</td>
<td>1088527</td>
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</tr>
<tr>
<td>Ascidia curvata (Traustedt, 1882)</td>
<td>17298</td>
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</tr>
<tr>
<td>Asciddia interrupta (Heller, 1878)</td>
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<td>21 m</td>
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<tr>
<td>Botryllodes nigrum (Herdman, 1886)</td>
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</tr>
<tr>
<td>Botryllus planus (Van Name, 1902)</td>
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<tr>
<td>Botryllus planus (Van Name, 1902)</td>
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</tr>
<tr>
<td>Clavelina oblonga (Herdman, 1880)</td>
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</tr>
<tr>
<td>Cystodytes dellechiacei (Della Valle, 1877)</td>
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</tr>
<tr>
<td>Cystodytes dellechiacei (Della Valle, 1877)</td>
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<tr>
<td>Didemnum conchyliatum (Sluiter, 1898)</td>
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</tr>
<tr>
<td>Didemnum conchyliatum (Sluiter, 1898)</td>
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<td>3-4 m</td>
</tr>
<tr>
<td>Didemnum conchyliatum (Sluiter, 1898)</td>
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<tr>
<td>Didemnum duplicatum (Monniot, 1983)</td>
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<td>Didemnum duplicatum (Monniot, 1983)</td>
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(Table 2 continued)

<table>
<thead>
<tr>
<th>Name</th>
<th>USNM #</th>
<th>Depth (in meters)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Didemnum duplicatum Monniot, 1983</td>
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<td>Didemnum vanderhorsti Van Name, 1924</td>
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<td>Diplosoma listerianum (Milne Edwards, 1841)</td>
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<td>Diplosoma listerianum (Milne Edwards, 1841)</td>
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<td>0-1 m</td>
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<td>Diplosoma listerianum (Milne Edwards, 1841)</td>
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<tr>
<td>Eudistoma cf. capsulatum (Van Name, 1902)</td>
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<tr>
<td>Eudistoma clarum (Van Name, 1902)</td>
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<tr>
<td>Eudistoma olivaceum (Van Name, 1902)</td>
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<td>Herdmania momus (Savigny, 1816)</td>
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<td>Lissoclinum fragile (Van Name, 1902)</td>
<td>20159</td>
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<td>Lissoclinum fragile (Van Name, 1902)</td>
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<td>Microcosmus exasperatus Heller, 1878</td>
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<td>Perophora formosana (Oka, 1931)</td>
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<td>1 m</td>
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<tr>
<td>Phallusia nigra (Savigny, 1816)</td>
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<td>0-1 m</td>
</tr>
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<td>Polyanandrocarpa sp.</td>
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<tr>
<td>Polycarpa spongabilis Traustedt, 1883</td>
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<td>Polycarpa spongabilis Traustedt, 1883</td>
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<td>Pyura vittata (Stimpson, 1852)</td>
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<td>Pyura vittata (Stimpson, 1852)</td>
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<td>14-15 m</td>
</tr>
<tr>
<td>Rhopalaea abdominalis (Sluiter, 1898)</td>
<td>19908</td>
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</tr>
<tr>
<td>Salpa fusiformis Cuvier, 1804</td>
<td>1014962</td>
<td>&gt; 5 m</td>
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<tr>
<td>Styela sp.</td>
<td>1014947</td>
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<td>Styela sp.</td>
<td>1014954</td>
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<td>Styela sp.</td>
<td>1088523</td>
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<tr>
<td>Symplegma viride Herdman, 1886</td>
<td>1014956</td>
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<tr>
<td>Symplegma viride Herdman, 1886</td>
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<td>&gt; 10 m</td>
</tr>
<tr>
<td>Trididemnum orbiculatum (Van Name, 1902)</td>
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</tr>
<tr>
<td>Trididemnum orbiculatum (Van Name, 1902)</td>
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</tr>
<tr>
<td>Trididemnum orbiculatum (Van Name, 1902)</td>
<td>1014948</td>
<td>0-1 m</td>
</tr>
</tbody>
</table>

- Lover’s beach: three species including one colony of *Aplidium*, three colonies of *Didemnum conchyliatum* and one colony of *Cystodytes dellechiajei*.
- Goat island in Batteaux bay: three species including one colony of *Aplidium exile*, one colony of *Didemnum duplicatum* and one specimen of *Ascidia interrupta*.  

Diversity and Distribution of Tunicata (Urochordata) in Tobago
Diversity and Distribution of Tunicata (Urochordata) in Tobago

- Bon accord lagoon: 10 species including two colonies of *Polyclinum constellatum*, three colonies of *Diplosoma listerianum*, two colonies of *Trididemnum orbiculatum*, two colonies of *Eudistoma olivaceum*, one specimen of *Phallusia nigra*, one colony of *Perophora formosana*, one specimen of *Styela*, one colony of *Symplegma viride*, one colony of *Botrylloides nigrum* and two colonies of *Botryllus planus*.

- Buccoo coral reef in Buccoo bay: nine species including four colonies *Didemnum cineraceum*, two colonies of *Didemnum vanderhorsti*, one colony of *Didemnum chonchyliatum*, two colonies of *Trididemnum orbiculatum*, two colonies of *Lissoclinum fragile*, 10 specimens/zooids of *Rhopalaea abdominalis*, one colony of *Eudistoma olivaceum*, four specimens of *Ascidia curvata* and eight specimens of *Polycarpa spongiabilis*.

- Pirates cove: one species—*Didemnum duplicatum* (one colony).

- Angel reef: one species—*Didemnum conchyliatum* (one colony).

- Petit trou lagoon: four species including two colonies of *Aplidiopsis stellatus*, five colonies of *Eudistoma olivaceum*, 32 specimens of *Styela*, 20 colonies of *Symplegma viride*.

- Pigeon point: one species—*Eudistoma olivaceum* (one colony).

- St. Giles (Melville) islands: 1 species—*Didemnum duplicatum* (2 colonies).

- Man-O-War bay: three species including nine specimens of *Microcosmus anchyloideirus*, four specimens of *Microcosmus exasperates* and 10 specimens of *Salpa fusiformis*, the only pelagic species found during the study.

- Booby point in Booby bay: six species including one colony of *Didemnum conchyliatum*, one colony of *Diplosoma listerianum*, one colony of *Trididemnum orbiculatum*, two specimens of *Herdmania momus*, eight specimens of *Microcosmus anchyloideirus* and two specimens of *Pyura vittata*.

- Brothers rock in Bloody bay: four species including one colony of *Clavelina oblonga*, one colony of *Eudistoma clarum*, one specimen of *Polyandrocarpa* and four specimens of *Microcosmus anchyloideirus*.

- Corvo point: two species including two colonies of *Cystodytes dellechiajei* and one specimen of *Pyura vittata*.

- North point: two species including five colonies of *Eudistoma capsulatum* and three specimens of *Microcosmus anchyloideirus*.

Table 1 shows Marble Island as a separated locality. It is actually located in the Man-O-War bay and the one species found there, *Microcosmus anchyloideirus* (4 specimens), was included in those of the findings of Man-O-War bay. The table makes note that tunicates exist on that particular island in the bay.

4. Tobago Fauna

The following tunicates were found at various locations on both the Atlantic and Caribbean sides of the island. Within a family, genera are listed in alphabetical order.

4.1 Class Ascidiae

4.1.1 Order Aplousobranchia

4.1.1.1 Family Polyclinidae Milne Edwards, 1842

Family characterized as having the body of zooids (individuals) in three parts: branchial (thoracic), abdomen and post-abdomen; gonads and heart are located in the post-abdomen. All species in this family are colonial.

(1) Genus *Aplidium* Savigny, 1816

This genus consists of elongated zooids; stomach has well developed plications (folds).

Species of this genus found in Tobago:

*Aplidium constellatum* (Verrill, 1871)—found at Lover’s Beach in Man-O-War bay. Habitat: coral and rock. It forms small colorless colonies. This species is common along the eastern US shelf, Atlantic Ocean, but not found everywhere in the Caribbean. It was, however, recorded by Goodbody [25] from Belize; and F. Monniot [21] from Guadeloupe.
Aplidium exile Van Name, 1902—found on Goat island in Batteaux bay (formerly Tyrrel’s bay) and was attached to dead coral. Collected from 3-4 m of water, colonies form small colorless colonies with somewhat flattened heads. Colony was found in a reef environment.

(2) Genus Aplidiopsis Lahille, 1890
Species of this genus have a smooth stomach and a constriction between the abdominal section and the post-abdomen.

Species of this genus found in Tobago:

Apliodiopsis stellatus Monniot and Monniot, 1984—colonies attached to mangrove roots in Petit Trou Lagoon on the Atlantic Ocean side.

(3) Genus Polyclunum Savigny, 1816
This genus has minute papillae in the branchial sac, the intestine is twisted into a closed loop, the post-abdomen is sac-like and separated from the abdominal section by a constriction.

Species of this genus found in Tobago:

Polyclunum constellatum Savigny, 1816—A small rounded colony was found attached to a mangrove root in Bon accord lagoon. This lagoon consisted of mangrove trees around its borders and tunicates thrived in the lagoon.

4.1.1.2 Family Didemnidae Verrill, 1871
All colonial, reproduce asexually by budding, zooids are small, body in two parts: the branchial (thoracic) and the abdominal. The abdominal part includes the gonads; some species have calcareous star-shaped spicules in the tunic.

(1) Genus Didemnum Savigny, 1816
This genus is characterized by having four rows of stigmata in the branchial sac and coiled sperm ducts around the testis, the tunic has spicules.

Species of this genus found in Tobago:

Didemnum cineraceum (Sluiter, 1898)—colonies were flat, soft, transparent and encrusted dead coral. Zooids can easily be seen through the test. This species was only found on Buccoo Coral Reef, none were found in the mangrove environments.

Didemnum conchyliatum (Sluiter, 1898)—found in the reef environments of Buccoo coral reef in Buccoo bay, Angel reef, Booby point and in Lovers beach and other parts of the Man-O-War bay. The colonies at Buccoo reef were large, flat, whitish gray and attached to dead acropora coral. At Lover’s beach, the colonies were dull white and much smaller than those at Buccoo coral reef and were attached to rocks.

Didemnum duplicatum Monniot, 1983—found in the reef environments of Goat island in Batteaux bay (formerly Tyrrel’s bay), St. Giles and Melville islands, Buccoo coral reef and an area just south of Pirates cove in the Man-O-War bay. The colonies were small and white, usually smaller than colonies of Didemnum conchyliatum.

Didemnum vanderhorsti cf. Van Name, 1924—light brownish transparent colonies found on dead coral in Buccoo coral reef. Colonies had very few spicules and zooids were large.

(2) Genus Diplosoma Macdonald, 1859
Genus characterized by four rows of stigmata in branchial sac, no coils around the divided testis, no spicules in the tunic.

Species of this genus found in Tobago:

Diplosoma listerianum (Milne Edwards, 1841)—common in the Caribbean. In Tobago, soft, fragile, gelatinous colonies were found closely attached to mangrove roots in Bon Accord Lagoon and on dead coral on Booby point and Man-O-War bay. In the field, colonies appear grayish.

(3) Genus Lissoclinum Verrill, 1871
Genus has four rows of stigmata in the branchial sac, no sperm duct coils around the divided testes, spicules in the tunic.

Species of this genus found in Tobago:

Lissoclinum cf. fragile (Van Name, 1902)—whitish transparent colonies were found attached to dead acropora coral at Buccoo Reef.

(4) Genus Trididemnum Della Valle, 1881
The genus has three rows of stigmata in the branchial sac, sperm duct coils around the testis, spicules in the
Diversity and Distribution of Tunicata (Urochordata) in Tobago

Species of this genus found in Tobago:

*Trididemnum orbiculatum* (Van Name, 1902)—white colonies were found growing on bivalves (mollusks) in Bon Accord Lagoon, also attached to dead coral on Buccoo coral reef in Buccoo bay, on the reef of Booby point and in Man-O-War bay.

4.1.1.3 Family Diazonidae Garstang, 1891

Family characterized by individuals having the body usually in two parts but some species have a post-abdomen containing the gonads and heart, branchial sac has no folds and has internal longitudinal vessels raised on supporting papillae.

(1) Genus *Rhopalaea* Philippi, 1843

Zooids are solitary with firm tunics, large branchial sacs, numerous rows of stigmata in branchial sac, abdomen section separated from thoracic section by a narrow neck.

Species of this genus found in Tobago:

*Rhopalaea abdominalis* (Sluiter, 1898)—collected from a depth of 10 meters by scuba. One clump of several zooids was found on Buccoo coral reef. Typical purple to maroon color with thick test.

4.1.1.4 Family Clavelinidae Forbes and Hanley, 1848

All colonial with translucent tunics, siphons are without lobes on short tubes and both are located at the anterior end of the body, branchial sac elongated with numerous rows of stigmata.

(1) Genus *Clavelina* Savigny, 1816

Zooids can be totally separated, partially embedded in tunic or totally embedded, numerous tentacles in the branchial siphon. Thorax (branchial sac) is large with no folds, four or more rows of stigmata, 20 or more stigmata per row.

Species of this genus found in Tobago:

*Clavelina oblonga* Herdman, 1880—one small colony collected from Brothers rock, Bloody bay.

4.1.1.5 Family Polycitoridae Michaelsen, 1904

All species of this family are colonial, body of zooids is divided into two parts (thorax and abdomen), zooids are usually entirely embedded in tunic, both siphons are 6 lobed, no folds in the branchial sac.

(1) Genus *Eudistoma* Caullery, 1909

Genus has three rows of stigmata in the branchial sac, small smooth walled stomach, the gonads and stomach are at the posterior end of the abdominal section of the zooid.

Species of this genus found in Tobago:

*Eudistoma capsulatum* (Van Name, 1902)—very small colonies, juvenile zooids from North Point.

*Eudistoma clarum* (Van Name, 1902)—one small clear glassy colony collected from Brothers rock in Bloody bay attached to a tubed polychaete.

*Eudistoma olivaceum* (Van Name, 1902)—a common colonial species of Tobago. This species was found in reef environments (Pigeon point and Buccoo coral reef) as well as in mangrove rich lagoons. On the reef, colonies were very dark green, very small, flat and colonies were widely separated from each other. In the nutrient rich lagoons (Bon Accord Lagoon and Petit Trou Lagoon), colonies grew larger.

In Pigeon point, a sandy beach environment, colonies were so closely attached to the coral or rocky substrate that they were difficult to remove without damaging the colonies.

In Bon accord lagoon, colonies attached to mangroves and were large and plentiful; here they grew on short stalks that were clustered together. The color of species in Bon accord lagoon was pale green.

In Petit trou lagoon, colonies were not stalked. They consisted of sometimes flattened, sometimes rounded olive green heads.

At Buccoo reef, the colonies formed stalked long slender olive green lobes attached to shells or solitary tunicates such as *Microcosmus exasperatus*. Many more colonies of *Eudistoma olivaceum* were found on Buccoo coral reef than Pigeon point because of the lack of sand on the former which allows a better flow of nutrients through the body.

(2) Genus *Cystodytes* von Drasche, 1884

The tunic usually consists of plate or disk shaped
calcareous spicules that sometimes completely enclose the zooids, tunic is firm, gelatinous and translucent with bladder cells, four rows of stigmata in branchial sac, thoracic and abdominal sections are closely connected with very little neck separating them, smooth walled stomach.

Species of this genus found in Tobago:

*Cystodytes dellechiajei* (Della Valle, 1877)—This is a common Caribbean colonial tunicate but only one small dark brown colony was found in Tobago; from Lover’s beach in the Man-O-War bay among coral and rock and Corvo point (just west of Man-O-War bay). It was not found in the mangrove habitats such as Bon Accord Lagoon.

4.1.2 Order Phlebobranchia

4.1.2.1 Family Ascidiidae Herdman, 1880

Family characterized with zooids with flat branchial sacs, usually no folds, numerous rows of stigmata and internal longitudinal vessels with papillae at their junctions with transverse vessels, branchial tentacles always simple.

(1) Genus *Ascidia* Linnaeus, 1767

Individuals are solitary, oval and elongated, firm translucent or semi-transparent tunic, laterally flattened body, no folds in the branchial sac, less than eight atrial lobes.

Species of this genus found in Tobago:

*Ascidia curvata* (Traustedt, 1882)—small transparent specimens were collected in 1958 during the Smithsonian-Bredin Caribbean Expedition from Buccoo coral reef and Booby point on the Caribbean side.

*Ascidia interrupta* Heller, 1878—a diver collected a specimen that was attached to a small rock in Batteaux bay (Atlantic side).

(2) Genus *Phallusia* Savigny, 1816

Large individuals, thick firm cartilaginous and usually naked tunic, dorsal ganglion posterior to dorsal tubercle, eight or more atrial lobes.

Species of this genus found in Tobago:

*Phallusia nigra* (Savigny, 1816)—observed by Kenny (1976) in Bon accord lagoon.

4.1.2.2 Family Perophoridae Giard, 1872

Colonial individuals, resemble small Ascidiiidae, zooids usually independent but joined by stolons, flat branchial sac, no folds, gut loop at left of branchial sac.

(1) Genus *Perophora* Wiegmann, 1872

Small individuals, round-ovate shape, individuals on short branch like stalks, transparent tunic, lobed siphons, muscles on mantle, gut loop across posterior part of the body, smooth stomach, gonads located in the gut loop.

Species of this genus found in Tobago:

*Perophora formosana* (Oka, 1931)—tiny colony found among other tunicates attached to a mangrove root in Bon Accord Lagoon. It is fairly common in the Caribbean but the author did not notice an abundance of this species among the mangroves in Tobago.

4.1.3 Order Stolidobranchia

4.1.3.1 Family Styelidae Sluiter, 1895

Solitary and colonial individuals, simple unbranched tentacles, usually only four folds in the branchial sac, dorsal lamina is a continuous membrane with no languets.

(a) Subfamily Styelinae Herdman, 1881. This subfamily consists of only solitary individuals.

(1) Genus *Polycarpa* Heller, 1877

Individuals have numerous small short hermaphroditic gonads located on the inner walls of both sides of the body, sometimes the gonads form rows and sometimes they are randomly scattered.

Species of this genus found in Tobago:

*Polycarpa spongiabilis* Traustedt, 1883—this grayish bulbous solitary species was collected during the Smithsonian-Bredin Expedition of the late 1950s by Waldo Schmitt from Buccoo coral reef.

(2) Genus *Styela* Fleming, 1822

Tunic opaque and leathery, both siphons are four lobed or square, four well developed branchial folds, gonads located on both sides of body, gonads consist of a tubular ovary (sometimes branched) and separate
male follicles attached to the ovary by ducts.

Species of this genus found in Tobago:

*Styela* sp.—small reddish globular individuals. This species was abundant, attached to the mangrove roots of Petit Trou Lagoon and a few individuals were found attached to the mangrove roots of Bon Accord Lagoon. The author did not find this species when I collected from Bon Accord Lagoon in Jan, 1993, but the abundance was observed in 2002 and 2006. This species will be described in a later paper.

(b) Subfamily Polyzoinae Hartmeyer, 1903

All individuals are colonial, never form colonial systems, individuals have siphons that open directly to the exterior of the colony (outside of the colony).

(1) Genus *Polyandrocarpa* Michaelsen, 1904

Characters similar to *Polycarpa* in that individuals have small numerous gonads on both sides of the body but species of this genus are colonial.

Species of this genus found in Tobago:

*Polyandrocarpa* sp.—one small lobe collected by divers from Brothers rock in Bloody bay.

(2) Genus *Symplegma* Herdman, 1886

Without folds, one gonad on each side of the body consisting of a central ovary and two testis follicles surrounding it, individuals are attached by their ventral sides with both siphons on the dorsal side.

Species of this genus found in Tobago:

*Symplegma viride* Herdman, 1886—some colonies were found on a mangrove root of Bon accord lagoon and several rather extensive colonies were found on the mangrove roots in Petit trou lagoon.

(c) Subfamily Botryllinae Adams and Adams, 1858

All species are colonial, individuals are completely embedded and arranged in systems, the male and female gonads are separate.

(1) Genus *Botryllus* Gaertner, 1774

Gonads on both sides of the body, ovaries are always anterior to the testis, embryos develop in the peribranchial cavity (the chamber surrounding the brachial sac).

Species of this genus found in Tobago:

*Botryllus planus* (Van Name, 1902)—attached to mangrove roots in Bon accord lagoon.

(2) Genus *Botrylloides* Milne Edwards, 1841

Individuals have a single ovary on each side of the body located posterior to the testis, eggs develop in a sac like pouch that develops as an outgrowth of the body wall.

Species of this genus found in Tobago:

*Botrylloides nigrum* Herdman, 1886—observed by Kenny in 1976 on mangrove roots in Bon Accord Lagoon.

4.1.3.2 Family Pyuridae Hartmeyer, 1908

Family consists of all solitary individuals with more than four folds in the branchial sac, straight stigmata, a tough leathery tunic that sometimes consists of spines, branched tentacles, digestive tract has a hepatic gland (liver), usually only one gonad on each side of the body.

(1) Genus *Herdmania* Lahille, 1887

This genus is characterized by having needle or rod-like spicules in the body wall and mantle.

Species of this genus found in Tobago:

*Herdmania momus* (Savigny, 1816)—two specimens were collected by divers from rock and coral on Booby Point and in Man-O-War bay also observed by Kenny (1976) in Bon Accord Lagoon.

(2) Genus *Microcosmus* Heller, 1878

Individuals have a smooth dorsal lamina with no languets, gonads on both sides of the body, the left gonad lies partly within the gut loop.

Species of this genus found in Tobago:

*Microcosmus anchylodeirus* Traustedt, 1883—several large dark red specimens were collected by scuba from Booby point, on a reef in Man-O-War bay, North point (east of Man-O-War bay), Brothers rocks in Bloody bay and Marble island in Man-O-War bay.

*Microcosmus exasperatus* Heller, 1878—large and small incrusted red specimens were found among gorgonians in Man-O-War bay. A grayish-beige specimen was found on a mollusk shell in Bon accord...
Diversity and Distribution of Tunicata (Urochordata) in Tobago

lagoon.

(3) Genus Pyura Molina, 1782
Individuals have a dorsal lamina that consists of languets, the left gonad does not lie across the intestine.
Species of this genus found in Tobago:

*Pyura vittata* (Stimpson, 1852)—grayish-beige specimen collected by divers from Booby point, in Man-O-War bay and Corvo point among gorgonians and rubble and attached to dead mollusk shells.

4.1.3.3 Family Molgulidae Lacaze-Duthiers, 1877
Solitary individuals, branchial sac has curved or spiral stigmata, branched tentacles, zooids have a large sac-like kidney.

No specimens of this family were found in Tobago but species *Molgula occidentalis* Traustedt 1883 does exist in the Caribbean sea.

4.2 Class Thaliacea
Family Salpidae Lahille, 1888
Pelagic, transparent, with atrial and branchial openings at opposite ends of the body.
Subfamily Salpinae
The tunic is often thick and firm, forming plates or spines, the gut is coiled.
Genus Salpa Forskal, 1775
Elongated and cylindrical individuals with dorsal and lateral body muscles.
Species of this genus found in Tobago:

*Salpa fusiformis* Cuvier, 1804-collected with a plankton tow net from Man-O-War bay.

5. Conclusion

As this work shows, Tobago has a fairly diverse population of tunicates, mostly colonial. Of the thirty two species collected for this study, approximate 80% were colonial sessile species, only one species was pelagic. In Tobago incrusting colonial tunicates of the family Didemnidae preferred coral reef habitats such as Buccoo coral reef and Booby point on the Caribbean side and Angel reef and Goat island on the Atlantic side. Solitary tunicates such as *Polycarpa spongiabilis*, *Microcosmus anchylodeirus* and *Herdmania momus* thrive on reefs as well. The habitats that seemed to be havens for both colonial and solitary species were the mangrove regions on both the Atlantic side (Petit Trou Lagoon) and the Caribbean side such as Bon accord lagoon. Bon accord lagoon was especially “rich” with tunicates. In this lagoon, one mangrove root can contain up to 10 different species. In 2000, the existence of *Molgula occidentalis* on the mangroves in the Caribbean country of Belize was reported by Goodbody [25] and the author had hoped to find this species in Tobago but no individuals were found during the collections noted in this paper. This study shows that even though Tobago is part of the southernmost island republic and just north of Venezuela, the island’s tunicate species are typically Caribbean on both the Caribbean Sea and Atlantic Ocean side.

Acknowledgments

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References

Effects of Counselling on In-School Adolescents’ about HIV/AIDS in Malaysia

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Abstract: The study examined the effects of counselling on in-school adolescents about HIV/AIDS in Malaysia. A total of 100 male and female students were randomly chosen from three residential commercial areas located in Kuala Lumpur in Malaysia. A questionnaire on HIV/AIDS symptoms was administered. The findings of the study indicated that two thirds of the interviewed adolescents have high desires to live longer on the earth. Also, adolescents do not associate high death rates of youths with HIV/AIDS symptoms. This report has implications for HIV/AIDS counselling in the Malaysian school system. This is supported by the fact that with the success of retrovirus therapy, many of the infected now live healthier lives and are pursuing a wider range of activities. Many HIV/AIDS infected people today can participate in undergoing their daily life activities, with a significant chance of living longer on the earth. The authors conclude that collaborative partnership between HIV/AIDS services and HIV counselling as part of the integrated system will provide early intervention services to boast relief of anxiety for the young people in Malaysia who already live with HIV/AIDS.

Key words: Adolescents, in-school counselling, HIV/AIDS.

1. Introduction

Acquired Immune Deficiency Syndrome (AIDS) is a serious and deadly disease caused by a virus that attacks and destroys the body’s defence system, thereby leaving the body vulnerable to any disease. The body becomes open to infection and disease which the body could normally fight off. It is expedient that AIDS poses a serious public health problem in South-East Asia. Malaysia has one of the fastest growing AIDS epidemics in the South-East Asia and Pacific region [1], and thus far, more than one-third (35.9%) of total 87,710 reported infections registered in the country were those who detected in younger people between the ages of 13-29 years. Mesweeney [2] pointed that AIDS is the greatest scourge of modern times, hence the most important new threat to the world health body. On the other hand, the Human Immunodeficiency Virus (HIV) according to Achau [1] is the causative organism which is the newest and most deadly virus. As Unachukwu [3] posited that, it was first identified in 1981 in the United States of America and 1982 in Uganda (East Africa). The intensive research and scientific activities conducted in various laboratories world-wide in 1983-1984 have led to the identification of a virus which causes AIDS. This virus which was previously given diverse types of names and has been officially named the Human Immunodeficiency Virus. The findings of the study will help the adolescents to understand that AIDS is a human disease that ravages the immune system undermining the body’s capability to defend itself against certain diseases leading to death. It will also help the counsellors to curtail the spread of HIV/AIDS among the teenagers through their counselling education programme. More importantly, the
government would help to assist trained counsellors in providing adequate information on HIV/AIDS. The adolescents could also understand that they are at high risk of HIV/AIDS because of their attitude towards sex.

This year, the Malaysian Ministry of Health [4] divulged a growing incidence of new HIV/AIDS cases through sexual transmission, with an increase in the number of new infections detected among young people (Table 1). In 2004, sexual transmission accounted for 20% of total cases; in 2008, this rose to 27% and the latest statistics shows that it is now 32%. There is a cause for concern as it is likely to rise, bearing in mind the estimated gestation period of 10 years of the virus, that new cases detected in persons below the ages of 30 would have been infected in their twenties and sometimes even during their teens.

Although HIV/AIDS was first associated with gay (homosexual) behaviour, in Malaysia the infection rapidly progressed into a phenomenon associated with intravenous drug use (IVDU). The main mode of HIV transmission today still is via drug-injecting equipment but this has been decreasing steadily and currently accounts for slightly over half (55%) of new infections last year.

Past nationwide surveys revealed that young Malaysians have been found to have uneven knowledge on HIV/AIDS and sexual reproductive health [5, 6] and even where knowledge is high, it was not being practiced [7, 8]. This has resulted in advocacy calls for a step-up in HIV/AIDS awareness campaigns targeted to educate the adolescents in Malaysia. Even a two-hour lecture campaign carried out at a college in the state of Perak, Malaysia, reiterated that perceptions, knowledge and attitudes of the adolescents increased after the intervention [9].

As in many Asian societies like Malaysia where traditional and conservative prevail, issues dealing with sex and sexually transmitted diseases are not discussed openly as it is considered impolite, taboo and sensitive. Zulkifli and Wong [5, 7, 8] on their part related that the era of the 1990s was strongly coloured by relaxation of social mores, social experimentation and juvenile and adolescent behavior dubbed as “lepak” (“hanging out”) and “bohsia” (which in the Chinese-Hokkien dialect means “nothing to do”). From “loitering” at public places such as malls and theatres, Zulkifli and Wong [5, 7, 8] submitted how this degenerated to activities in streets and secluded places which led to sexual contact. While these behaviors were highlighted in the press, the major concern are for parents and the government, they were also the social effects that come with liberalisation and modernisation.

### Table 1  Results of premarital screening in 2009.

<table>
<thead>
<tr>
<th>Age</th>
<th>Male</th>
<th></th>
<th>Female</th>
<th></th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No. Screened</td>
<td>No. confirmed +ve</td>
<td>No. Screened</td>
<td>No. confirmed +ve</td>
<td>No. Screened</td>
<td>No. confirmed +ve</td>
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<td>0</td>
<td>0</td>
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<td>61</td>
<td>0</td>
<td>63</td>
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<td>31,823</td>
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<td>54,466</td>
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<td>14</td>
<td>31,632</td>
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<td>30-34</td>
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<td>8,546</td>
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<td>337</td>
<td>0</td>
<td>1,703</td>
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<tr>
<td>Total</td>
<td>91,517</td>
<td>42 (0.05%)</td>
<td>87,751</td>
<td>17 (0.02%)</td>
<td>179,268</td>
<td>67 (0.04%)</td>
</tr>
</tbody>
</table>

Source: Ministry of Health (2010)
Effects of Counselling on In-School Adolescents’ about HIV/AIDS in Malaysia

on a society dominantly bound by traditional family values and societal sanctions. So, despite a predominantly Islamic society in which pre-marital sex is unlawful, adolescents in Malaysia’s multi-religious and multi-ethnic population do date, have “steadies” and many engage in unsafe sexual intercourse [10].

The incidence of adolescents engaging in sexual intercourse also increases with age [11]. Even in an earlier study [5, 7, 8], it was estimated that 20% of 1,181 unmarried respondents aged 15-21 years old reported having had sexual activity. What is more alarming is that most sexual encounters are unsafe, with no protection against sexually transmitted diseases and unwanted pregnancy WHO [12]. Unsafe sex is a major threat to the health and survival of adolescents. In 2005, over 70,000 adolescent girls were admitted to public hospitals in Malaysia with close to 37% for pregnancy and related problems [13]. Hence, with their vulnerabilities towards drug-taking and sexual relationships, there is no doubt that In-school adolescents are at greater risk of acquiring HIV/AIDS than other age groups. With no specific cure for HIV/AIDS, preventive measures based on information and education programs remain crucial for tackling HIV/AIDS and its associated problems [14-16]. Thus, communication and intervention strategies play important roles in educating the public, especially the young adult, on the prevention and control of HIV/AIDS.

Recent studies have shown that many infected adolescents in Malaysia do not know about the pandemic, which is the important implications for the prevention of ailment through information. The risk of HIV transmission among individuals is likely to increase. Infected individuals choose to have sex without considering the implications so that it is certainly necessary to educate the young people who are prospective leaders of tomorrow.

Consequent upon the stigma attached to HIV/AIDS and the victims in Malaysia, there is no national demographic survey for the purpose of examining the impact of HIV/AIDS as it affects the youngsters and the counselling implications. This work will provide an insight into such trends in Malaysia.

No one could have thought 25 years ago, when the first cases started to make its ugly appearance that HIV prevention would be as difficult as it has proven to be. Malaysia is no exception, and especially when it comes to HIV prevention advocacy for adolescents (14-19 years). As prevention is better than cure, the Health Ministry in its strategic planning efforts for HIV programmes in 2009 top-listed as priority the issues of adolescent vulnerability in sexual transmission of HIV affecting school going and out-of-school youth. The subject of sex education or reproductive health (under which HIV prevention comes), however is still virtually non-existent to adolescents in educational institutions, with Malaysian Ministry of Health [4] statistics revealing only a 0.2% penetration of such programs, and that too, a pilot project in schools. This is owing to a number of issues including cultural taboos and religious norms regarding matters related to sex-education, political will in what is considered a “sensitive” issue, parental objections, the lack of resources (manpower and finance) and the competency (training) of available resources. In fact, there is no specific budget set aside by the government for the education sector, for the ministries involved in education and even for young people for the multi-sectoral activities towards HIV/AIDS prevention.

Most programmes targeted at youth are done on an ad-hoc basis and such is the situation despite studies having shown that school-based HIV programs are effective in reducing risk-taking behaviors [17]. Adolescents in this country, after all, are a “captive” audience as schooling is compulsory in Malaysia from the age of six, and albeit a small proportion of those who for some reason cannot attend school or drop out, majority remain in school until forms five, between 17 and 18 years old. Most programmes addressed at adolescents thus far have either been carried out by non-governmental organisations and, as mentioned
earlier, are considered ad-hoc and are very seldom conducted on school premises.

Most recently in July this year, following a spate of social problems “besieging youth such as teenage pregnancies, abandoned babies, unsafe abortions and sexually transmitted diseases” [18], the Malaysian Government announced its intentions to formally introduce Reproductive and Social Health Education (which includes HIV/AIDS awareness) as a core subject in the school curriculum. Hopefully this will get off ground in view of the fact that HIV/AIDS poses a serious threat to young people. Thus far, there have been very few specific studies targeted solely on in-school adolescents in the Federal Territory of Kuala Lumpur. Most researches in this area has been on a nationwide basis or on a different target population group.

What are the effects of counselling on the in-school adolescent as to avert the HIV/AIDS pandemic ravaging the world population? How can the stakeholders in all spheres of human endeavour be meaningfully mobilized in the scourge of this disease? How can the in-school adolescents who dwell in the more modern, urban setting of the capital city of Kuala Lumpur where there is better infrastructure and easier access to information via technology and development be educated about sex?

2. Materials and Methods

This study was carried out in Kuala Lumpur, Malaysia, with the purpose to improve the quality of life of in-school adolescents. It consists of providing counselling services to young people using a variety of qualitative research approaches including semi-structured interviews and short questionnaires. We conducted a qualitative questionnaire survey in three areas of Kuala Lumpur, and in handing out 50 modified questionnaires. Based on those validated in previous studies [5-8, 19], we received 43 fully completed survey forms. The randomly-selected respondents were between 14-19 and surveyed in three residential-commercial areas of Kuala Lumpur, Bangsar, Taman Tun Dr Ismail and Desa Hartmas, all in the vicinity of fast-food establishments. These surveys and interviews were carried out at both private and public spaces depending on the respondent’s request. The questionnaires were both in English and Bahasa Malaysia, the main languages used in schools, and consisted of 25 main questions on:

- General HIV/AIDS knowledge (5),
- Modes of HIV infection (5),
- Prevention of HIV infection (6)
- HIV testing (2)
- Self-efficacy (7)

Ranging from “Yes/No” answers to ticking of boxes for correct/incorrect answers as well as to indicate preferences on the four open-ended questions. Responses were scored 1 if correct and 0 if incorrect or unanswered. The overall score was calculated by adding the scores from each of the 5 sections of the test. Possible overall scores ranged from 0 to 32. The knowledge of availability of HIV/AIDS-related health services in the country was also queried, whereby respondents were asked where to seek HIV/AIDS related testing, treatment, and counselling or advice. The survey took between 15 and 20 minutes to be completed.

3. Results and Discussion

Of the 50 survey questionnaires handed out, we received 43 fully completed ones i.e. 86% response rate. Demographically, there were 23 from females and 20 from males. Ethnically, the respondents were Malays (16 professing Islam), Chinese (12 Buddhist and Christian), Indians (nine Hindus and Christian) and others (six Christian and Buddhist). Majority came from families earning RM3,000-RM4,000 monthly. All 43 respondents had heard of AIDS and while most of the older respondents (16 to 19 years) cited first learning about it from newspapers and Television, the younger ones (14 to below 16 years) cited the Internet as their first source. In fact, majority
subsequently cited the Internet and friends as the “other sources” they had learnt more about HIV/AIDS.

However, when asked about Question 5 (Do you know the difference between HIV and AIDS?), 28 of the respondents (65%) knew the difference between the virus which leads to the disease. As to whether it was possible for a healthy-looking person to be infected, 38 of the respondents (88%) of them were well aware, and quite a few cited the recent case of popular German girl band singer Nadja Benaissa, 28, who was found guilty for causing bodily harm to her ex-boyfriend by having unprotected sex with him despite knowing she was infected with HIV. On August 26 this year, Benaissa was given a two-year suspended prison sentence and 300 hours community service. She had faced a possible 10 years behind bars [20].

Over 88% (38 participants) knew there was no cure for AIDS, and knew about blood-testing for the virus and were aware that early detection could prolong life. However, most of them—40 respondents (93%) were not aware or had heard of anonymous HIV testing, which has been made available in the country. Majority received their information from the Internet followed by TV and the newspapers. This is consistent with findings in the other countries [5, 7, 8] where the majority of young people appeared to rely on the public media (television and newspapers) as their primary source of HIV/AIDS information. However, our survey revealed that Internet has overtaken these media [9, 21]. Also consistent with other studies [22] it was that a relatively high percentage of young people did not receive information from family members and medical professionals.

Most of those surveyed knew sharing injecting needles, having sexual intercourse with an infected person or with multiple partners with unknown HIV status and receiving a transfusion of infected blood could cause the HIV virus to be passed on. However, 40% were unsure that receiving an organ from an infected person (91.9%) was a mode of transmission while many did not know that the virus could be passed from an infected mother to her foetus (85.6%). Most were aware that casual contact, toilet seats and swimming pools cannot transmit the virus. However, a smaller majority were aware that tattooing and piercing (63.3%) and the sharing of personal items (60.8%) are mode of transmission.

About 38 of the respondents (88.3%) believed that HIV/AIDs could be prevented, listing the most well-known modes of prevention as:

- to avoid taking drugs,
- not share injecting needles and syringes, and
- to have sex with only one faithful uninfected partner.

However, a smaller majority, i.e., 27 respondents (62.7%) were aware that HIV/AIDS could be prevented by using condoms.

Majority of the respondents knew that blood tests (94.7%) could be used for detecting HIV infection, but fewer were aware that DNA tests (68.3%), urine tests (63.0%) and oral fluid tests (48%) can also be used for testing. Again the majority noted that they could obtain HIV testing (94.6%), and treatment from government hospitals but nearly half were not aware of HIV-related counselling (50.2%).

As to the question: How much are you at risk of contracting HIV/AIDS, all 43 felt ticked the box “No risk at all”. This was backed by all also saying “Yes” to Question 24 on “Would be useful to develop a personal game plan” of what to do to avoid getting infected with the HIV/AIDS virus? However, on the elements to be included in the game plan, 16 respondents (37%) did not tick the “use of condoms” while 38 respondents (88%) did not feel it necessary for both partners go for HIV voluntary testing.

The present study has shown the persistent increase in the rate of promiscuity among the adolescents. For instance, Onyemelukwe [23] reported high sexual permissiveness among adolescents and Enemuo [24] reported the same high permissive attitudes among adolescents which show no change in behaviour. But the situation would have been a different thing if the adolescents have real knowledge of the HIV/AIDs.
Ignorance is really a disease. The much they know could not make them think towards the avoidance of this scourge. It is necessary to state here that healthy carrier may exist and will continue to share the disease with other uninfected people. The overall scores revealed that slightly more females had more knowledge about HIV/AIDS than the males. This is in tandem with findings in other studies on gender differences in AIDS knowledge [5, 7, 8, 25]. Of the ethnic groups, the Malays (16) respondents were found to be more knowledgeable [5, 7, 8, 26]. On the whole, results from this qualitative survey indicated that the respondents had high knowledge of HIV/AIDS with a mean score of 26 (81.25%) out of a maximum 32 points (calculated by adding the scores from each section of the questionnaire). There was also a significant correlation that those with lower household incomes had lower scores while those with the highest incomes had the highest scores. Generally, knowledge on HIV/AIDS transmission and prevention was inaccurate. However, there were some misconceptions about condom use as well as an indication of not wanting to go to the Government institutions or health clinics for further information, as in the case of other studies [5, 7, 8]. This situation of young person’s being “overconfident” has also been found in other studies [8, 26]. Of concern is the finding that high knowledge score is independent of sexual behaviour and practices, that is, knowing all about HIV/AIDS may not necessarily be protective against infection [7, 8]. Similarly, gender differences in perceptions, beliefs and attitudes, particularly with regards to sexual behaviour, are also important findings.

Our extrapolation is that the adolescents in Kuala Lumpur may have a high theoretical perspective of the virus and disease but a more in-depth “realistic” perspective is lacking. None of the respondents had ever seen or known a HIV/AIDS patient personally or seen local pictures of victims, only foreign ones. In fact, many of the respondents mentioned identifying with the popular HIV/AIDS awareness MTV “Staying alive” campaign (http://www.staying-alive.org/en/) which can be watched over television as well as the Internet as an ideal programme which could be “localized” in content. Also for example, what of the situation posed by migrant workers of which there is a large population in Malaysia? Naturally, there are bound to be relationships between the mainly male foreign workers and local partners. As an indication that the adolescents would like to know more, all respondents again felt that Reproductive and Social Health Education should be part of the school curriculum (as in Question 22) with most ticking the answers (Question 23) that it promoted responsible behaviour and was a child’s right.

The general view of adolescents interviewed was that knowledge tends to be more “academic”, bordering on the superficial. For example, while aware of general modes of transmission, many said they were not aware of specific modes of transmissions (such as tattooing). A good number also expressed confusion over myths and misconceptions about the disease which perpetuates stigma and discrimination against those who have contacted HIV/AIDS (such as not having a meal with an HIV-infected person). Hence, there is the obvious need to educate adolescents and teach them life skills such as negotiation, conflict resolution, critical thinking, decision-making and communication, and improve self-confidence and the ability to make informed choices, such as postponing sex until they are mature. In other words, putting knowledge into practice, adolescents interviewed said that it was time for the issue of HIV/AIDS to “really come out of the closet” so that those at-risk populations like themselves could have easy access to accurate knowledge through schools, communities and the media.

Some aspects of HIV/AIDS are not known or understood by adolescents and neither do they have access to any informative nor training materials on the disease. Hence, there is a need to improve materials in terms of content easily digestible by the adolescent...
age-group and these should be widely available. For example, printed materials could be in the form of comics, contain humor, be entertaining, and be produced indicating youth sections of newspapers or magazines for young people in our school system. As for electronic media, video games are already making inroads with positive results achieved from the use of interactive games in educating youth on health behaviours such as HIV/AIDS prevention [1, 5]. This, after all, is an extremely “mobile” generation where the Internet is accessible via mobile phones and other new-technology gadgets.

Generally, professionals involved in HIV/AIDS advocacy are of the opinion that while general knowledge of adolescents in the urban areas such as Kuala Lumpur can be high, especially in comparison with those in the rural areas, there is no reinforcement of these perceptions in “real life” situations. Calls have been made to promote “life skills” into the school curricula. Increasing services and coverage and reinforcing coordination among government, community and international agencies and NGOs will undoubtedly strengthen and help win the fight against HIV/AIDS in our country [27].

HIV/AIDS advocacy programs are apparently not as effective as hoped for and this is shown by not a single respondent having ever heard about the Ministry of Health’s PROSTAR program (Healthy Living without AIDS for Youth). The Malaysian AIDS Council (MAC) has continuously been advocating reducing the vulnerability and infection among young people (age 15-24) by the creation of a supportive environment for HIV prevention. Malaysian AIDS Foundation chairman Prof. Dr. Adeeb Kamarulzaman said that prevention, stigma and discrimination are the three main issues for Malaysia in dealing with HIV/AIDS. On projects specially targeted at youth, the MAC’s goals are to:

1. establish the MAC Youth Wing to focus on youth issues.
2. empower youth leaders to coordinate and sustain HIV/AIDS programmes
3. increase youth access to life skills-based education and accurate information on HIV/AIDS.

Adeeba reported that HIV infection among women had increased from 9.4% in 2000 to 19.1% in 2008, indicating a need for HIV/AIDS advocacy to be addressed towards adolescent females. However, in our opinion, these programmes as well as others by various NGOs have limited reach as attested to by the growing incidences of young people being new detected with HIV [4].

Founder and past president of MAC, Marina Mahathir in her fortnightly “Musings” column in the Star Newspaper [28] reiterated that calls for comprehensive sex education in our schools had long been made but to no avail, despite the rise in teen pregnancies and in sexually transmitted diseases, including HIV.

In 2009, there was an increase in marriages involving underage Muslims in Kuala Lumpur; 49 Muslim girls under 16 years of age and 39 boys under 18 tied the knot [29]. This goes against the assumption that child marriages are now on the decline due to changing cultural trends. According to the statistics provided by the Federal Territory of Religious Department, this number was higher compared with the previous year (Under Islamic family law, only girls and boys aged at least 16 and 18 and above respectively, can marry but the syariah court can grant permission for younger children to marry). What is more about pre-marital screening for HIV/AIDS? The table gives a statistical indication of the situation in Malaysia.

It is appropriate to go a long way as to introducing an effective sex education module in school, but we should all start thinking about it not only in the light of the risk posed by HIV/AIDS, but also to educate our adolescents about the risk of teen pregnancies, STDs, as well as coping with the challenges that emerge from the shaping of one’s sexual identity.

The question is that, can behavioural change alone affect the future HIV scene. Undoubtedly, the answer
lies in understanding the fact that HIV/AIDS is a complex involving meshed factors at individual, group, societal and country level [7, 8].

The Malaysian Ministry of Health’s Public Health Institute has developed a training module for counsellors involved in the management of the HIV infection. They include health care workers, counsellors working in prisons, drug rehabilitation centres and the religious departments.

Counseling in this arena is an active process of communication and dialogue between a trained counsellor and a client with a problem related to HIV or AIDS, and with a view to dealing with the issue adequately and appropriately. Among the various objectives of counselling are:

1. Prevention of infection through promotion of healthy life styles, behaviour, moral and spiritual values.
2. Prevention of transmission through modification of risky lifestyles and behaviours.
3. Provision of psychosocial support to those infected and/or affected by HIV/AIDS to achieve optimum level of functioning and satisfactory quality of life.
4. To complement health education and correct misconceptions or myths about HIV/AIDS.

Adolescents are a special focus group for counselling for the following reasons:

Many think that they are not at risk of HIV and that HIV testing is for sick people; Young people do not want others, especially their parents to know that they have been tested for HIV;

- Peer pressure, lack of assertiveness, low self-esteem, poor sexual identity, risk taking, boundary-setting and limitations, substance abuse, sexual exploration, poor family relationships, sexual abuse and domestic violence, pregnancy and unsafe abortion, STI/HIV disclosure, and lack of family planning are all issues that affect young people making them vulnerable to getting infected with HIV.

There are some general principles that should be observed when counselling adolescents. Counsellors should try to ensure that all the information given during the sessions, especially regarding their sexuality is understandable and appropriate to the mental and emotional development of the adolescent. In maintaining confidentiality, the adolescent client should also be given an explanation as to its limits in regard to sexual abuse, suicidal tendencies among others. This means that at some point, depending on the severity of the problem, the counselor may have to discuss it with someone else, especially if the situation is life threatening or outside the law.

HIV/AIDS counsellors are trained to discuss the aspects of normal growth and development, how they affect an adolescent’s view of life, the influence of risk-taking behaviour and the tendency to rebellion. They remain open to dialogue around the adolescents’ current issues of concern and areas of misinformation while exploring the teenager’s skill levels, especially in such areas as decision-making, using vocabulary that they will understand.

Counselors must be aware of appropriate community practices (i.e., what the community does when they have addressed a problem beyond their present means, e.g., dealing with a violent drug-addict or a person who makes a young girl pregnant), and when appropriate, to make referrals to someone who has more knowledge or expertise on specific issues that come up in the counseling sessions.

Adherence to (or in some cases, compliance with) either medical or psychological plans that have been set up during the sessions are strengthened if there is an ongoing, trusting adolescent-counselor relationship. Adolescents should have a mandate to know their HIV status. They should be fully informed to appreciate consequences for many aspects of their health, including sexual behaviour. The approach to all should be in line with culturally acceptable practices. Health care workers should also encourage adolescents to involve their parents in their care, if this is culturally appropriate.
4. Conclusion

From the analysis and the discussion of the findings, the following conclusions could be made: It is obvious that adolescents are not aware of the aetiology of HIV/AIDS.

It is also now clear that most of the adolescents are not aware of the epidemiology of HIV/AIDS and the adolescents are not aware of the clinical presentations of HIV/AIDS.

There is serious and urgent need for psychologists, the guidance counsellors, medical practitioners and social workers to mount a grassroots campaign on the existence of HIV/AIDS for children and adolescents. Psychologist and Guidance counsellors in Kuala Lumpur in particular should start organizing group guidance programmes to educate the adolescents on HIV/AIDS pandemic in Malaysia.

The timely challenge is therefore to establish an efficient teaching-learning procedure, including counselling module geared towards imparting HIV/AIDS preventive knowledge to adolescents. As Zulkifli [7, 8] stated that, there remain gaps and misconceptions which need to be addressed.

Counselling services should definitely be an integral part in the management and prevention of HIV/AIDS and this has been suggested to be available in schools by a counsellor or even a health care nurse, at least three times a week [30-31].

There is also a need to expand the telephone counselling services at the national, state, and local levels to help those wanting to know more about the virus and disease, especially adolescents.

These young people, such as our survey respondents, may have a high level of theoretical knowledge but they also have the right to realistic and practical youth-friendly information, skills and services for HIV prevention at all stages of their formative years. Only with a positive attitude towards managing their lives will these adolescents stand a better chance of avoiding the pitfalls of a society where drug addiction and casual sexual relations lurk.

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