Investigation on *Agrilus nubeculosus* (Fairm) with Special Reference to its Association with Gummosis in *Acacia Senegal*

Sayeda Ali Ahmed Khalil[1]

Abstract

This study was carried out in El Heimayra forest (Northern Kordofan State) to investigate farmer's believe that there is a positive correlation between the abundance of *A. nubeculosus* and the yield of gum arabic from *A. senegal.* Trees from three age classes namely; 5-9, 10-14 and 15-20 years old were selected randomly. Twenty trees were covered with wire mesh and sprayed with 57% malathion and labeled as control trees (C) and 20 other trees were left for natural infestation by the insect and labeled as infested trees (O). These experiments were replicated during three tapping periods: early, mid and late. Yield from infested trees increased by 97% in all age classes and tapping periods. Yield from infested trees was highest in early period in all age classes. Yield from infested trees varied considerably in its physical properties (colour, shape, optical rotation, moisture content and size) as well as chemical properties (nitrogen, ferrous, calcium and manganese contents) as compared to gum from control trees. The life cycle of *A. nubeculosus* was investigated. Micro-organisms in the hindgut of *A. nubeculosus* were isolated and identified as *Aspergillus* spp.and *Staphyllococus* spp. Trees which were inoculated with fungus produced higher amounts of gum than those which were inoculated with fungus and bacterium simultaneously, with *bacterium* alone, and higher than trees which were naturally infested with *A. nubeculosus* and control trees. It is apparent that *A. nubeculosus* transmits both *Aspergillus spp.*and *Staphylococcus spp.*which might have presented a stress that resulted in more gum production confirming earlier hypothesis that gummosis is a pathological reaction and/or a modification of some physiological processes of *A. senegal* as a result of microbial infection.

Introduction

Many gum producing farmers believe that gum yield is directly correlated with abundance of a certain beetle locally known as "Garraha" and that in years when the beetle population is high, gum production is usually high and vice versa (Hassan, 1999). There is no clear evidence so far to describe production of the gum (gummosis) of *A. Senegal*. However, scientific proof is lacking and many of these theories are hypothetical. Several gum producers interviewed in Kordofan firmly believe that a beetle locally called "Garraha" attack the tree making holes into the newly created wounds on tapped gum trees and that the gum exudes only from those holes. Holes are made by pushing a sharp posterior organ into the fresh wound tissues of the tree, secreting a dirty and greenish

[1] National Forestry Corporation

material over the area. The farmers are certain that the presence of these insects mean good gum production, and that their absence means the opposite. This assertion seems worth investigating (El khalifa, *et al*, 1989). Many theories have been put forward to explain the formation of gum. As early as 1926, Blunt believed that it is a product of the unhealthy state of the tree and that gum is the form in which the tree manufactures its food (Osman, 1993). Malloy (1972) stated that, gum arabic is accelerated by bacterial infection. He hypothesized that a celluolytic fungus induces gum production, when isolating the fungus and injecting it into the trees; he claimed that production was increased tremendously with a marked reduction in cost. Awouda (1973) indicated that The process of gum formation may not be due to unhealthy state of the tree but rather due to adverse environmental conditions under which the tree is living, these conditions are mainly poor soil, low rainfall and high temperatures. Both of these theories may explain partially the process.

Hypothesis

This research is based on the hypothesis that El Garraha beetle transmits a certain bacteria and/or fungus on tapped area of *A senegal* and that the activity of this bacteria and/or fungus affects exudation of gum Arabic.

Materials and Methods

Study Area

The study was carried out in El-himaiyra Forest (12 Km North to El-Obied). Its lies between longitude 13°-19` O5s N and 30°- O9` 58s E at 545m asl.

Impact of the beetle

The first experiment was designed to study the impact of the insect on gum production by *A. senegal* trees. A total of 40 healthy (5-9 years old) *A. senegal* trees (24.75 cm mean diameter and 2.68 m mean height) were selected randomly in El-Himaiyra forest plantation. In each tree, two branches were selected randomly and tapped (19 Nov. 1999). The tapped area ranged between 100 cm x 7-5 cm – 70 cm x 8-6cm, the tapping was done with the tool called locally sonky. Of the 40 trees, 20 were left open to natural infestation by El Garraha and the remaining 20 trees (at the opposite site of the natural infestation trees in the forest) were sprayed with 57% malathion before tapping and covered with a wire mesh to prevent the entrance of the beetle. The second and third tapping were carried out in mid and late periods (13/2/2000 and 11/4/2000, respectively). After 30-40 days the gum was hand picked, weighed and kept in polythene bags inside a refrigerator at 10 ± 1 °C for further analysis. The gum from open and control trees was collected and kept separately.

The same experiment was carried out for 10-14 and 15-20 years old trees with a mean diameter of 33.2 cm and 39.9 cm, and mean height of 3.4 m and 4.3 m, respectively. The collected gums were analyzed for chemical and physical characters.

SAS computer package was used for statistical analysis and separation of means.

Determination of the Cationic Composition of Gum:

The cationic composition of gum from both control and infested trees was determined according to AOAC (1984). According to this method, one ml extract from each sample of gum was dissolved in 50 ml of distilled water in a conical flask. Three drops of NaOH, with a small amount of peroxide indicator were added. Calcium, magnesium, manganese, iron, and phosphorus, in the diluted extracts were determined volumetrically by titration against EDTA.

Life Cycle of El Garraha

The second step was to study the life cycle of the beetle. A total of 10 adults of El Garraha were captured and kept in strong paper container together with healthy branches of *A.senegal* for one year. In another container, some infested branches were kept with 10 adults of El Garraha for six months. The biology, shape and life cycle of the various developmental stages of El Garraha were carefully observed and recorded with the aid of a binocular microscope.

Isolation of Micro-organisms in the Hindgut of El Garraha

Media Preparation (Preparation of Blood Agar)

The 3ird study was to isolate micro organisms from the hindgut of the insect. Isolation was done on blood agar medium. This medium was prepared by dissolving 28.2g nutrient agar in one litre of distilled water in a two litre Erlenmeyer flask, the flask was plugged tightly with a piece of cotton and autoclaved for 15 minutes at 121^0C and 15 p.s.i. 100 ml of sterile melted nutrient agar medium was cooled to 48^0C, and then 10 ml of sterile defibrinated blood was added and mixed gently to avoid bubbles.

The medium was poured while warm into sterilized petri-dishes in a laminair cabinet and left to solidify. The plates were then incubated at room temperature for 24 hours, as described by Collins *et al*, (1995).

Three adults of El Garraha were washed three times with sterile distilled water. Then the contents of the hindgut were pressed into sterile Petri- dish, which contained 1ml of sterile distilled water on a laminair cabinet. A loopfull of the suspension was streaked on the blood agar plates. All cultures were incubated in the laminair cabinet at 32 ± 2 °C for 24 hours.

Fungus identification

The fungi was cultured in PDA medium, which was prepared by dissolving 39 g of PDA in one litre of distilled water in a two litre Erlenmeyer flask, and autoclaved for 15 minutes at 121^0C and 15 p.s.i. The medium was poured into sterilized Petri-dishes and left to solidify at room temperature at 32 ± 2 °C for 24 hours. The emerging fungi were grown on Czapek dox agar (CZ) medium, (Kulwant,*et al*, 1991) with the following ingredients; Sucrose =30.0 g, $NaNO_3$ = 3.0 g, $K_2 HPO_4$ = 1.0 g, KCL = 0.5g, $MgSO_4.7H_2O$ = 0.01g, Fe $SO_4.7H_2O$) = 15.0g, Agar = 15.0 g, Distilled Water = 1000 ml, Trace metal solution = 1.0 ml

For three-point inoculation, approximately 0.5 ml of molten agar (0.2%) and detergent (0.05%) were taken in a small vial with screw cap, and sterilized. A needle was

used for transfering the conidia to Petri dishes which were incubated at 25°C for 7 days in complete darkness in an upright position.

The colony characters were recorded and slides were made in lactic acid for morphological characters to be observed through a compound microscope.

Characterization of bacterial isolates

The bacterial colonies were transferred from blood agar medium plates to the nutrient agar for identification. The following microscopic test was performed on the bacterial isolates: Gram staining of 24 hours old nutrient cultures was performed as described by Collins *et al*, (1995). A loopfull of bacterial colony from the slanted stock culture was transferred and mixed with a drop of sterilized distilled water at the centre of a clean glass slide. The resulting suspension was spread with a sterilized loop to obtain a thin film and was left to air- dry. It was then fixed by passing over a flame 5-9 times. The smear was flooded with crystal violet-ammonium oxalate complex (prepared by dissolving 2 g of crystal violet in 20 ml of 95% ethanol and 0.8g of ammonium oxalate in 80 ml of distilled water). The two solutions were mixed and left to stand for 24 hours and then filtered for one minute. The excess dye was washed off with gentle running tap water. The smear was then covered with gram's iodine solution (prepared by dissolving 2 g of potassium iodide and 1g of iodine in 300 ml of distilled water) for one minute and then washed under running tap water. The smear was decolorized with 95% ethanol until no more stain came out. It was then washed with tap water and counter stained with safranine dye (prepared by adding 0.25 g of safranine to 10 ml of ethanol then made up to 100 ml with distilled water) for 30 seconds. Excess safranine was washed off with running tap water, and smears blotted dry and finally examined microscopically under oil immersion. Cells, which were violet coloured were recorded as gram-positive, those which were red were recorded as gram-negative.

For shape determination**,** the same technique used for the determination of gram reaction was employed for determining the cell shape.

Biochemical test *(Oxidase test)*

Oxidase test. Two ml of methyl phenylenediamine dihydrochloride (10%w/v aqueous solution) was added to a filter paper in a sterile Petri-dish. Using a platinum loop a heavy amount of growth from a 24 hours old culture (grown on nutrient agar) was added to the filter paper and quickly spread. Development of a purple colour within 10 seconds was recorded as a positive reaction for the presence of cytochrome oxidase (Collins *et al,* 1995).

Inoculation of *A. senegal* trees by Bacterium and Fungus:

Fifty trees of *A. senegal* were selected randomly to be inoculated with micro-organisms. All trees were tapped by sonky. The isolated bacterium was grown in nutrient broth, while the fungus was grown on PDA broth in conical flasks. Inoculation was carried out by taking 10 ml of either suspension and then sprayed on every tapped area of the branch using sterile syringes. Then the inoculated branch was covered with a piece of cloth. Firstly, 10 trees were labelled as group (B) and inoculated with the isolated

bacterium (later identified as S*taphylococcus aureus)*. Secondly, 10 trees were labelled as group (F) and inoculated with the isolated fungus (later identified as *Aspergillus flavus*). Thirdly, 10 trees were labelled as group (FB) and inoculated simultaneously with *A. flavus* and *S. aureus.* Fourthly, 10 trees were labelled as group (N) and left for natural infestation. The remaining 10 trees were labelled as group (C). The tapped area in group (C) was covered with a piece of plastic and served as control. Three replicates were taken and averaged.

Results and Discussion

Classification and General Description of El Garraha:

The beetle called El Garraha *(local name*) was identified and confirmed by the Natural History Museum in United Kingdom as:

Class**:** Insecta, Order: Coleoptera, Family: Buprestidae, Genus: Agrilus, Species: *Agrilus nubeculosus* Fairm

A. nubeculosus is an elongated beetle and measured about 8–9 mm, brightly coloured and with metallic sheen of violet.

The Life cycle of *A. nubeculosus*:

The Eggs. The adult lays white spherical eggs on dead branches. These eggs were clustered and white in colour. After two weeks the eggs hatch, and the larvae emerge.

The Larva. There are two larval instars: The first is 4-6 mm long, white with a black spot in the middle of the head, its legless and has a wide prothorax and flattened head. The body is slender with a small head; broad prothorax and tapering towards the end of the abdomen. This stage took about 2.5-3 months to change to the second instar. The second instar larva has a black head and 3 pairs of projections, elongated body, with a white creamy colour, and measured 5-7 mm long. It is a typical Buprestid larva. The larva tunnels into the wood of dead branches.

The Pupa. The first pupal stage is white creamy. 5-7 mm and after three days the colour changed to bright brown and the antennae and body segmentation were apparent. This stage took 15-20 days to develop into the imago and feeds on dead wood and measured about 6-8mm long.

The Imago. This phase has two stages: A-The first stage emerged after 3 days; the colour is brightly light brown, about 6-8mm long and feeds on dead wood (powder from tunnels). This stage took 3 days to develop its wings, legs and sense organs. B- The second stage is about 7-9mm long, brown with fully developed appendages).

The Adult stage. The adult is elongated and about 8-10mm long, brightly colored, the elytra were metallic sheen of violet. The adult emerged immediately in autumn, and it appears on tapped areas of hashab branches . The adult feeds mainly on green plants and fleshy fruits. The adults appeared after tapping immediately, and alighted on tapped areas of branches for two days and left their faeces on the tapped area. The adults lay their eggs on small felled branches. *A. nubeculosus* has two generations per year.

Appearance of *A. nubeculosus* **on Hashab Trees**

Eight hours after tapping, about 2-3 insects rested on the tapped area, and were very active on newly tapped areas of trees. They stayed for about two days after tapping, during which mating took place. After two days they left the trees, and a greenish white rounded spot was observed on tapped area.

The Impact of *A. nubeculosus* **on Gum Yield**

As shown in Table 1, gum yield from infested trees varied significantly (p= 0.0001) in different seasons of tapping (early, mid, and late). The maximum yield per tree was produced in early season, the yields per tree in mid and late were the same and lower than that produced in early season. In all age classes gum yield per tree increased as a result of natural infestation by the beetle (Table 1). In control trees, gum yield per tree in all age classes was not significantly different, when comparing early, mid, and late yield.. These results showed that the control trees were not affected by periods at different age interval.

Isolation of micro-organisms from the hindgut of ***A. nubeculosus***

A fungus and a bacterium were dominantly isolated from cultures.

Description of the fungus:

1. Colony diameter: 4.0 to 4.5 cm
2. Obverse: Yellowish - green becoming green with age.
3. Reverse: Creamish – yellow
4. Head: Radiating, becoming loosely columnar with age.
5. Stipe: long, verrucose, hyaline
6. Vesicle: Dome - shaped, fertile on entire surface.
7. Metulae: Present, small
8. Phialidae: small, ampulliform
9. Condia: Gulobose to subglobose, usually rough, yellowish-green.

According the above description the fungus was identified as *Aspergillus. Flavus* (Link.)

Description of the bacteria:

1. Shape: Cocci
2. Gram stain: Positive
3. Oxidase test: Positive

According to the above description and tests carried out the bacterium was identified as *Staphylococcus aureus*(Ros.)

Physical properties of gum from infested and control trees:
Description:

1. Colour: Gum from infested trees has a red colour and that from control trees is white creamy.
2. Shape: Gum from infested trees was spherical or round shaped, whereas that from control trees has an irregular shape.

3. Size: Gum from infested trees is bigger in size than that from control trees. Moisture Content: The moisture content of gum was significantly different at (P = 0.019), comparing the gum from infested trees and control trees (Table 2).
4. Viscosity: There is no significant difference in viscosity percent comparing the gum from infested trees and control trees (Table 2).
5. Specific Rotation: The mean specific rotation of gum was highly significantly (P= 0.001) different comparing gum from infested and control trees (Table 2).
6. Ash: The mean ash content was not highly significantly (P= 0.122) different comparing gum from infested and control trees (Table 2).

Chemical properties of gum from infested and control trees

As shown in Table 2, nitrogen, ferrous, calcium and magnesium contents were significantly higher in gum samples from infested tress than in samples from control trees, whereas the phosphorous and manganese content were not significantly different in samples of infested trees and no-infested trees.

Inoculation of *A. senegal* trees:

The application of *A. flavus* and *S. aureus* separately and in combination significantly (P=0.0001) affected gum yield per tree, (Table 3). Mean gum yield per tree was 249.7 g, 144.3 g, 136.2 g, 60.5 g and 0.0 g in trees inoculated with the fungus, fungus and bacterium, naturally infested with *A. nubeculosus*, bacterium and control trees, respectively (Table 3).

When comparing the gum yield from inoculated and naturally infested trees, the gum yield per tree increased by 83%, 6%, 56%, and 100% for fungi, combination of fungi and bacteria, bacteria and control tress, respectively.

Economic value:

It is believed that the insect is able to transmit pathological infection (fungal and/or bacteria) to healthy trees subjecting them to stress. To increase gum production and yield, efforts have so far concentrated on expansion of tapped forests and plantations. Unfortunately, many of those attempts were not successful. Tapping of trees is hard physical work that mainly takes place during the time when water is in short supply. Excessive tapping shortens the live of the tree. Moreover, the decrease and irregularity of the supply of gum arabic to the international market have caused many of the users to shift to other, cheap alternatives. Farmers believe that the presence of the "Garraha" insect “is a favorable environmental conditions conducive for gum arabic production.

The insect affect gum yield per tree, and can promote gum production. Gum yield per tree in trees that were inoculated with fungi was higher. These results study can be used to improve gum production in the Sudan through biological methods. Efforts to augment local knowledge with elements of scientific knowledge are likely to prove fruitful in increasing gum yield per tree, reducing the damage on trees and widening the range of technology options. Results of inoculation could contribute to poverty reduction among the poorest people and to increase export incomes.

Conclusions

Based on the results of this study it can be concluded that the gum yield per tree for one picking was 250 g for trees treated with the fungus, 60 g for trees treated with the bacteria and 144 g for trees treated with fungus + bacteria.

Recommendations

1. Use of biological method to increase the gum yield per tree by increasing the abundance of the beetle in the gum belt (rearing and distribution of the beetle).
2. Further investigations in the contamination of gum produced by the biological methods.

References

Collins, C. H.,patricia, M.L. and Grange, J.M. (1995). Collins and Lyne`s Microbiological Methods 7th edition 493 PP, Butterworth Heinmann Ltd. Linacre Hourse, Jordan Hill, Oxford OX2 . 8DP.

El Khalifa,M.D. Samani, M. O. E. Abdel Nour, H. O. *et al* , Gum Arabic rehabilitation in The Republic of The Sudan, Stage I Report, Volume 2: Main Report and Recommendation for Action, 1989.Institute of Environmental Studies , University of Khartoum, Sudan.

Hassan, R.A.(1999) Investigation of Stem Cutting Propagation and Factors Affecting Gum Production of *Acacia senegal* (L) WILD. M.Sc. Thesis 1998, Thesis, Department of Siliviculure, Faculty of Forestry, University of Khartoum.

Malloy, M.J.(1972) Unpublished paper, on Gum Arabic research in Ethiopia, 1972.

Osman, M. E. William, P. A, Menzies, A. R. and Phillips, G. (1993). Characterization of commercial samples of gum arabic. Journal of Agricltural Food chemistry. 41,71,77.

Osman, M. E. (1993). Characterization and Fractionation of the Gum from *Acacia senegal*. PhD thesis, Salford University. United Kingdom.

AOAC (1984) Officials Methods of Analysis, Association of Official Analytical Chemistry, 14th . Edn. Washington

Table 1. Average yield of gum (g per tree) in the three age classes at different priods

Period	5-9 years		10-14 years		15-20 years	
	Control	infested	Control	infested	Control	infested
Early	3a	102a	2a	123a	2a	146a
Mid	3a	141b	2a	118a	2a	146a
Late	2a	99a	2a	110a	1a	111b

Table 2. Compositional differences between infested and control gum

Elements %	Control	Infested	Probability
Moisture	8.7	7.6	0.19
Viscosity	1.3	1.5	0.23
Optical Rotation	-29.4	-33.2	0.001
Ash	2.8	3.1	0.122
Nitrogen	0.35	0.30	0.013
Ferrous	1.56E-03	1.66E-03	0.028
Phosphorous	0.23	0.23	0.18
Calcium	0.41	0.50	0.06
Manganese	8.167E-05	6.133E-5	0.06
Magnesium	7.900E-02	4.200E-02	0.001

Table 3. Gum yield from inoculated and control trees

Treatment	Mean(gm)
Fungi	249.66a
Bacteria + Fungi	144.33b
Control (natural)	136.19c
Bacteria	60.53d
Treated trees	0.00e

Means with the same letter are not significantly different.
LSD = 3.1002