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Egg incubation of *Achatina fulica* on humidified absorbent cotton and the identification of the fungi around the unhatched eggs

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Key words: Eggs, hatching, absorbent cotton, *Aspergillus Penicillium*

1 SUMMARY

The objective of this study is to show that cotton is a good incubation substrate for the *Achatina fulica* eggs. The egg incubation of *Achatina fulica* (giant African snail) on humidified absorbent cotton gave an average hatching rate of 71.09 %. It can thus be regarded as a good substrate for egg incubation of *Achatina fulica* following sawdust (90%). However the growth (weight) of snails during the five months of hatching was slow it was higher than the average of the starting (63.66g) .The study of the fungi contamination of eggs shows that the rate of contamination of unhatched eggs lies between 0.73 and 12.13 %. The contamination by *Aspergillus Niger* was highest at (12.13 %) and the weakest by *Mucor sp.* at (0.73 %) the percentages of appearance of fungi on unhatched eggs were as follows: *Aspergillus Niger* (40.74 %), *Aspergillus terreus* ((13.18 %)), *Mucor sp.* (2.46 %), *Penicillium sp.* (12.34 %)), *Phoma sp.* (18.51 %) and *Trichoderma sp.* (12.34 %). *Mucor, Penicillium, Trichoderma* and *Aspergillus terreus* were the least frequent and the least abundant. Among these moulds, at least two kinds (*Aspergillus* and *Penicillium*) were potentially toxinogenes.

2 INTRODUCTION

Absorbent cotton or teased cotton has achieved unanimity for its purity and its unequal softness. It is a natural and biodegradable crop product marketed after carding and chemical treatments like bleaching which removes from its fibers the resinous and fatty substances. In snails, the age, the size or the live weight of the individuals influences much the growth (Zongo et al, 1990). It even seems that, for all the species taken together, the speed of growth in the first months of the life would stabilize about the sixth month (Daguzan, 1983). The species *Achatina fulica* adapts very well to all kinds of environment. It modifies its biological cycle according to the local conditions. But, when the temperature or moisture is not appropriate to them, they enter in dormancy. The non hatching or the low level hatching of the eggs of *Achatina fulica* on various incubation substrates in particular on absorbent cotton, have been studied (Agongnikpo, 2003), (Dedi, 2007). The percentage of unhatched eggs on sawdust, plantation soil, virgin forest soil, coconut husks and absorbent cotton) could be because they are parasitized, desiccated or not fertilized (Mateo, 2006).

The fungi contamination is the leading cause of non hatchability of eggs. At the time of the

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contamination of eggs, the parameters controlling the fungi growth and which allow the production of toxins are numerous. There is mainly the initial load in microflora; the water content and the temperature of incubation of eggs are named. The increase in the temperature and or moisture can cause appearance of small maggots which attack the seed oysters and eggs; that leads to the putrefaction of the substrates with a considerable reduction of the rate of hatching.

The objective of this work was:
The incubation of the eggs (15) of *Achatina fulica* in sterile Petri dishes on humidified absorbent cotton, to determine of the hatching rate of the eggs of *Achatina fulica* and the percentage of unhatched contaminated eggs, to determine of the average weight of snails throughout the study, to identify the stocks of fungi which develop around unhatched eggs and their percentage of appearance.

3 MATERIALS AND METHODS

3.1 Biological material: There were three laying (eggs) per by *Achatina fulica* at a rate of 315, 350 and 340 eggs per laying. 945 eggs of the 1005 laid eggs were used. The evaluation of the hatching rate of eggs was carried out simultaneously with the evaluation of the percentage of appearance of fungi around unhatched eggs and of the rate of contamination of unhatched eggs.

3.1.1 Snails: There were fourteen mature animals (*Achatina fulica*) collected only once in the experimental plantations of the University of Abobo Adjamé. They were set out in two vats at a rate of seven animals per vat (is a density of 50 snails per m^2).Their average live weight was 63.66 ± 1g with an average length of shell of 8.40 ± 0.3 cm. The genital opening of the animals presented a light projection of whitish color, a sign of their sexual maturity (Ategbo et al, 2000)

3.2 Breeding: This study required the use of vats of chestnut color, in the form of pyramidal trunk of 65 cm length and 43 cm width for the great base; 43 cm of length and 36 cm of width for the small base and 20 cm height. The protection device consisted of a rectangular framework out of wooden 65 cm length and 52 cm broad covered with stainless iron netting. The bottom of the vat was furnished with litter in sufficient quantity made up of a mixture of brown ground of sand and clay coming from the forest of the university of Abobo Adjamé and sandy soil approximately quartz 87% (lagoon soil) which allowed snails to hide. The litter was sterilized with the autoclave with 121°c for 30 minutes then wetted with sterile distilled water. The vats for breeding rested on a rack one meter from the ground. The snails were nourished with 180g of lettuce (Lactus sativa: Apiaceae) each two days. Every two days, the vats are cleaned, food renewed, the detected layings are delicately taken using a spoon and put to incubate on absorbent cotton soaked with sterile distilled water at a rate of 15 eggs per sterile Petri dish. The snails are weighed every two weeks in order to appreciate their ponderal evolution throughout the period of the breeding. The determination of the live weight was carried out after the snails which had been showered, washed down, came out of their shells.

3.3 Food : The distributed food was made up of fresh lettuce deposited on the litter. The choice of this vegetable was guided by the fact that it is very rich in water (92%) and contains many minerals which reach a rate of 720 Mg for 100 G. Potassium leads with 234 Mg (more than 30% of the total of minerals) follow-up by the calcium (37 Mg) which supports the growth of snails best.

3.4 Hatching rate of eggs : The number of hatched eggs was given by counting the number of seed oysters.
The hatching rate is calculated

$$\text{According to the formula: } TE\ (\%) = \frac{NR}{NO} \times 100$$

TE = Rate of Hatching, NR = number Seed oysters
NO =number eggs

3.5 Fungi isolates: The isolation of the moulds was carried out by depositing 15 eggs of *Achatina fulica* taken randomly in the laying in each of the three sterile Petri dishes containing the absorbent cotton soaked with 5 ml with sterile distilled water. In general, the majority of fungi were mésophiles with optima of growth of 25-35°C (Botton et al, 1990). The relative humidity which is regarded as the crucial factor necessary to the fungi proliferation supports the growth of fungi starting

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from a water content of 65% (Botton et al, 1990). The eggs cleaned before with sterile distilled water were covered with wet cotton (Fig. 1)and the petri dishes prepared for incubation (Fig. 2) at an average temperature of 28°C in darkness for 10 to 15 days with an average rate of moisture of 82.2%. After this time the unhatched eggs presenting a development of moulds were put separately in sterile Petri dishes containing a sterile culture medium and Potato Dextrose Agar (PDA) MOREAU C, 1991). The seed oysters (hatched eggs) (Fig. 3) were counted. After 5 to 7 days of incubation in the darkness and with 28, 28 °C, the visible colonies were identified according to (Chabasse et Bouchara, 1997), (Champignon, 1997), (Bouree, 2001). For each experiment, the analysis was carried out under three tests at a rate of three repetitions per test.

The percentage of appearance of fungi is calculated according to the following formula:

$$\% \text{ fungi appearance} = \frac{\text{No. of appearance of fungi}}{\text{Total no. of appearance of all the fungi (81)}} \times 100$$

The rate of contaminated unhatched eggs is calculated according to the following formula:

$$\text{Rate of unhatched eggs contaminated} = \frac{\text{Unhatched no. contaminated}}{\text{Total unhatched egg no. (272)}} \times 100$$

Figure 1: Eggs of *Achatina fulica* lay out on absorbent cotton humidified in a Petri dish

Figure 2: Incubation of eggs of *Achatina*

Figure 3: Egg seed oysters of *Achatina fulica* hatched on absorbent cotton afterwards 10 days of incubation

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3.6 Treatment of the results: The statistical analysis of the values was carried out with software SPSS Duncan test enabled us to make a comparison of the average weights of snails. The difference between the averages is considered statistically non significant with the threshold of 5% (p = 1 > 0, 05). The comparisons between the three tests of the same experiment and between the tests of the seven experiments were made by a variance analysis to a criterion of classification (ANOVA 1). The software used was STATISTICA 6.0.

4 RESULTS

The rate of hatching remained higher throughout the experiments (Fig. 4) than the rate of unhatched. The best rate of hatching (85.17 %) was obtained with the third experiment and the low level (58.51%) with the first (Table 1). The highest ate of unhatched (41.49%) was obtained with the first experiment and the small percentage with the third (Table 1). Throughout the study, the average weights of *Achatina fulica* went increased then reached maximum average weight (78g) at the 16th week (Table 2, Fig. 5). At the end of the fungi analysis, 5 different kinds could be isolated and identified (fig. 6 to 11) from unhatched eggs. Thus, the *Aspergillus* kind is represented by two different species (*A.niger, A. terreus*). The kinds *Trichoderma, Mucor, Penicillium* and *Phoma* are represented by an isolate each. *Aspergillus Niger* is present at 40.74% and *Aspergillus terreus* 13.58 % on unhatched eggs. The kinds *Trichoderma*, *Mucor*, *Penicillium* and *Phoma* are respectively present at 12.34, 2.46 %, 12.34% and 18.51% on unhatched eggs (Table 3). *Aspergillus Niger* and *Aspergillus terreus* are respectively present on 12.13 % and 4.04 % of unhatched eggs. *Trichoderma*, *Mucor*, *Penicillium* and *Phoma* are present on 3.67 %, 0.73%, 3.67 % and 5.51 %of unhatched eggs (Table 3). 15 unhatched eggs were identified 5.51 % of eggs around which no mould had developed during the incubation period and even beyond that. These eggs remained intact on cotton keeping their white color which indicated that they were healthy.

Table 1: Rate of hatching, non hatching and the duration of incubation of eggs of *Achatina fulica* on absorbent cotton

Experiments	1	2	3	4	5	6	7
Rate of weights of hatching (%)	58.51 ± 5.04	79.25 ± 2,20	85.17 ± 2.81	71,10 ± 3.77	62.95 ± 3.72	65,91 ± 1.83	74,80 ± 1.92
Rate of weights non-hatching (%)	41.49 ± 5.04	20.75 ± 79.25	14.83 ± 2.81	28.90 ± 3.77	37.05 ± 3.71	34.09 ± 1.83	25.20 ± 1.92
Duration of incubation (d'ays)	12	11	11	10	10	10	10

An experiment: it is the whole of 3 tests at a rate of 3 repetitions per test. Cumulated average rate: 71.09 % on absorbent cotton

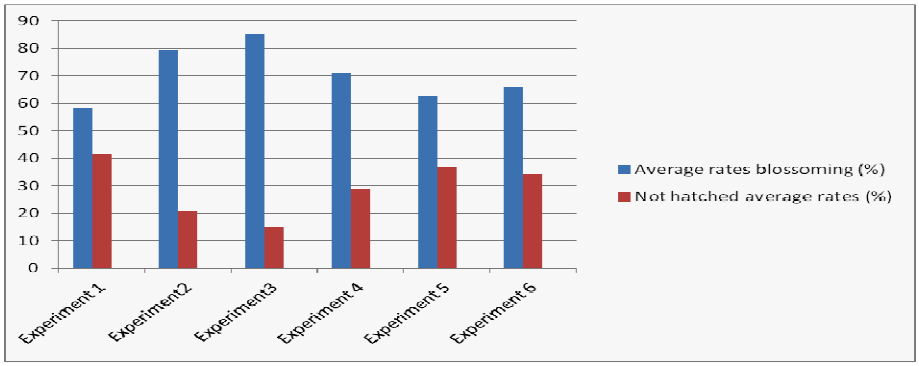

Figure 4: Diagram of the rate of hatching and non hatching eggs of *Achatina fulica* on sterilized absorbent cotton

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Table 2: Average weights of the *fulica* over five months of breeding

Achatina fulica **Dates**	**Average weights (g) of the A *fulica***
02 – 01 – 07	63.66 ± 2.25
16 – 01 – 07	65.66 ± 5.27
30 – 01 – 07	64.66 ± 5.46
14 – 02 – 07	67. 6 ± 8.68
28 – 02 – 07	73 ± 8.98
14 – 03 – 07	70.83 ± 14.56
28 – 03 – 07	71.50 ± 17.74
11 – 04 – 07	67.33 ± 10.89
25 – 04 – 07	78 ± 12.23
09 – 05 – 07	71.16 ± 23.72
23 – 05 – 07	76.16 ± 29.73

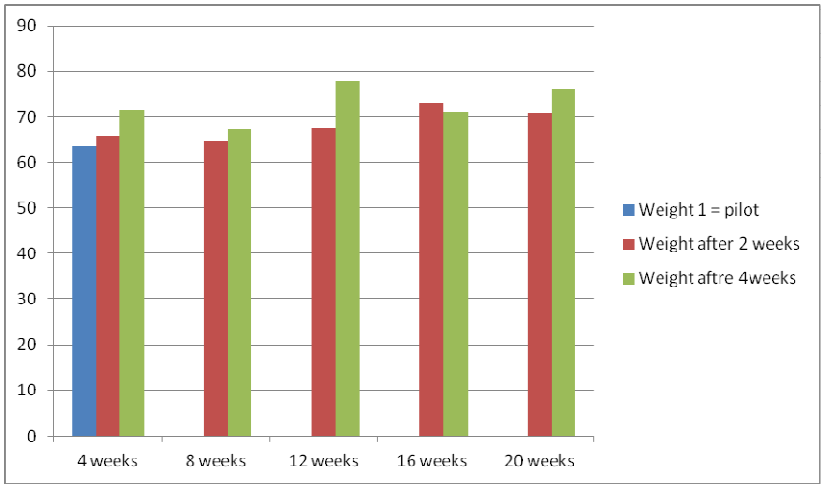
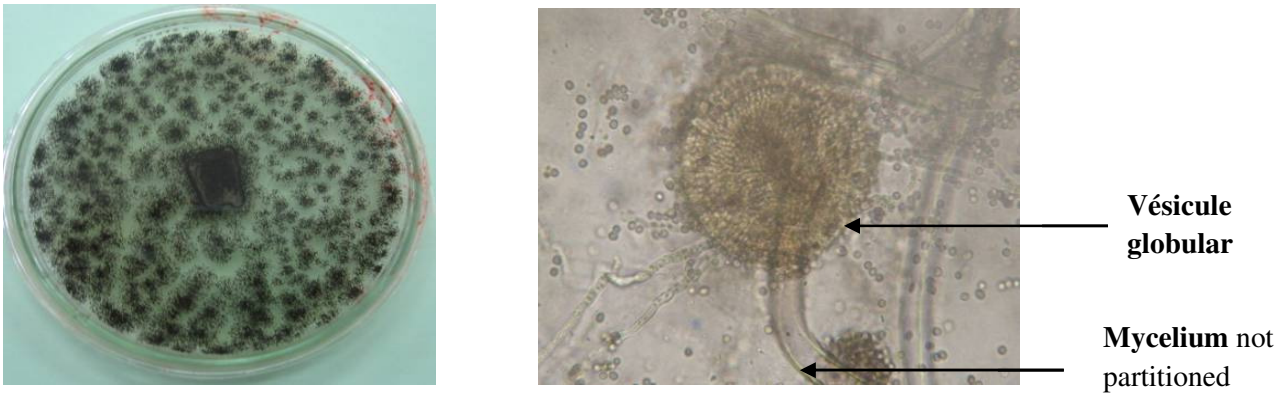


Figure 5: Diagram of the average weights of the snails *Achatina fulica*

1.2



a: Macroscopic aspect of *Aspergillus niger* **b** :Globular blister presence mycelium not partitioned (G x.400)
Figure 6: Macro and microscopic structure of *Aspergillus Niger*

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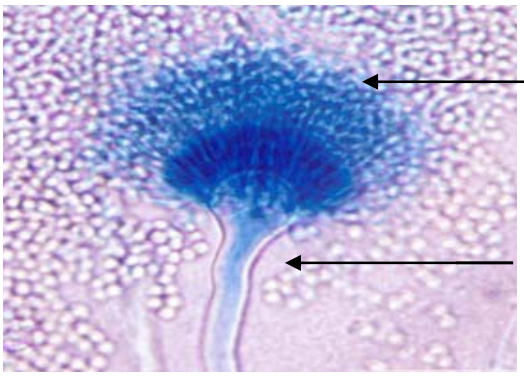
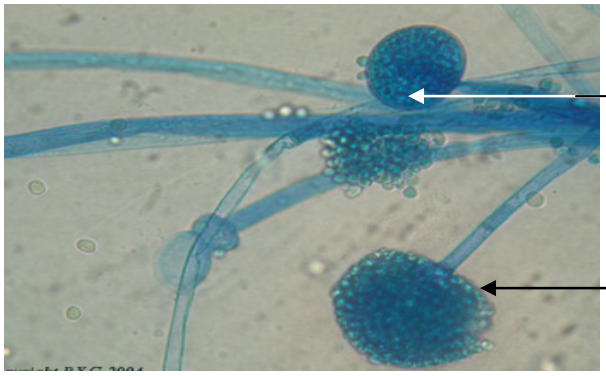
1.08cm



a: Macroscopic aspect of *Aspergillus terreus* **b** : Final blister, long compact with many conidies (Gx400)

Figure 7: Macro and microscopic structure of *Aspergillus terreus*

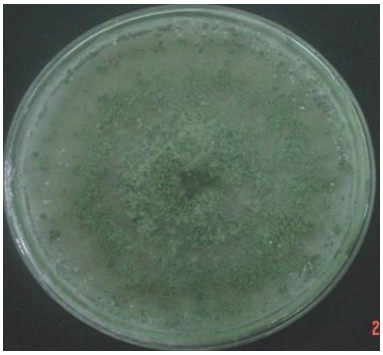
1.5 cm

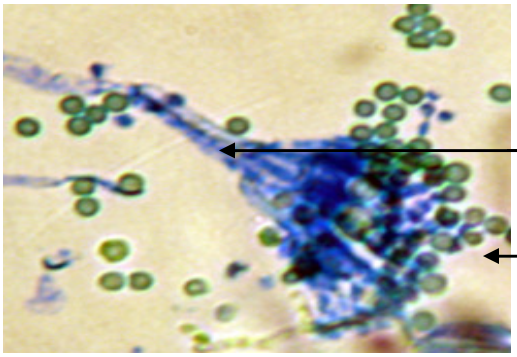


a: Macroscopic aspect of *Mucor sp.* **b:** Globulous sporocysts carried by trapped thalli (G x.400)

Figure 8: Macro and microscopic structure of ***Mucor sp.***

1.1cm





a: Macroscopic aspect of *Penicillium sp.* **b**: Many round conidies ramified, conidiophore (G x.400)

Figure 9: Macro and microscopic structure of ***Penicillium sp.***

1.1cm

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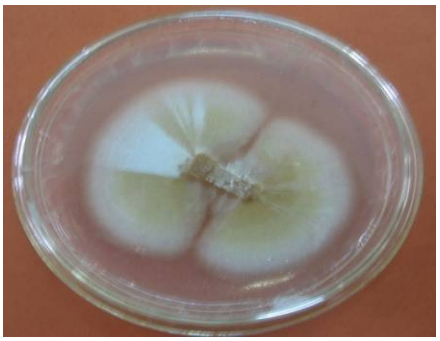
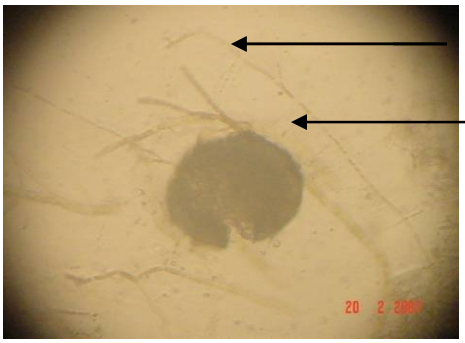
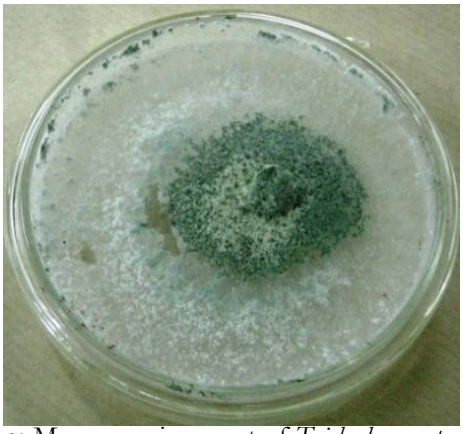


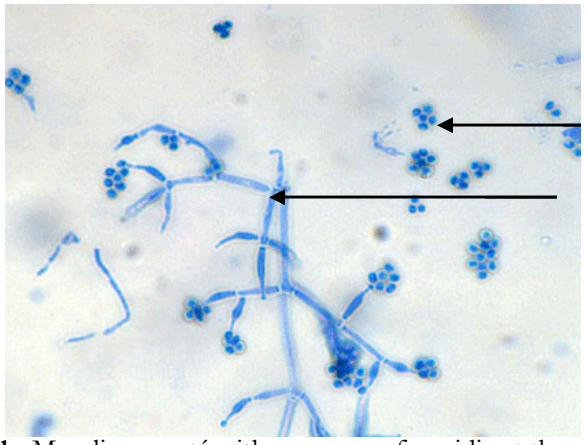


a: Macroscopic aspect of *Phoma sp.* b: Pycnide burst with mycelium partitioned (G x.400)

Figure 10: Macro and microscopic structure of ***Phoma sp.***

1.3 cm





a: Macroscopic aspect of *Trichoderma sp.* **b:** Mycelium septé with presence of conidie at the end of the phialides (G x.400)

Figure 11: Macro and microscopic structure of ***Trichoderma sp.***

Table 3: Percentage of appearance of fungi and rate of unhatched contaminated eggs

Number of appearance of Fungi	% of appearance	Rate of unhatched contaminated (%)
Aspergillus niger 33	40,74 ± 1,20	12,13 ± 1,20
Aspergillus terreus 11	13,58 ± 0,61	4,04 ± 0,61
Mucor sp. 2	2,46 ± 0,17	0,73 ± 0,17
Penicillium sp. 10	12,34 ± 0,40	3,67 ± 0,40
Phoma sp. 15	18,51 ± 0,64	5,51 ± 0,64
Trichoderma sp. 10	12,34 ± 0,57	3,67 ± 0,57

5 DISCUSSION

The rate of hatching on the whole of the experiments was higher than the average with a maximum with the third experiment (85.17 %). There was no significant difference between tests 1 ($p = 0.152 > 0.05$), 2 ($p = 0.1080 > 0.05$), tests 3 ($p = 0.722 > 0.05$) of the seven experiments carried out. However, there is a significant difference between: test 1 and tests 3 and 2 of the experiment 2 ($p = 0.000322 < 0.05$); test 3 and tests 1 and 2 of the experiment 7 ($p = 0.011 < 0.05$). The average rate of hatching to each experiment and cumulated average rate 71.09 %) were higher than the rate of hatching obtained by (Agongnikpo , 2003) (35.93 %) (Dedi , 2007) 39.51 %) after incubation of the

whole of eggs of a laying in a vat. It thus should be announced that the fact that the eggs are packed is not always advantageous. This fungus (*Fusarium oxysporum*) if it is present, contaminates the embryos of snails in the eggs which caused the disease called pink laying. When this fungus is present, it changes the white color of eggs to gray or pink. These eggs are desiccated or rotted and ended up contaminating healthy eggs. Although the impact of this disease is low. Nevertheless the substrate where the snails lay their eggs must be disinfected with boiling water for extracting the fungi. The fungi such as *Aspergillus, Fusar*ium and *Penicillium* can produce mycotoxins which could act on the rate of hatching eggs. There is on cotton substrate a rapid of hatching of the eggs (10 days) because the fibers of cotton are fine, light, resistant absorb water and restore moisture; it is thus a good conservative of moisture as the sawdust which prevents eggs from drying and breaking. But too much moisture supports the development of moulds. According to (Codjia and Noumovi , 2002) the rate of hatching of *Achatina fulica* is better on the sawdust (90% on average). (HODASI, 1979) 77,30 % of the rate of hatching at two weeks of incubation obtain on absorbent cotton. The present study on average gives a cumulated average rate from71.09 % to 10.57 day of incubation. These two rates are good and close to that obtained on the sawdust. Absorbent cotton can also be to regarded as one of the best substrates for incubation of eggs of *Achatina fulica* because of the speed that the eggs hatched. 5.51 % of unhatched eggs did not present any development of fungi nor of putrefaction of the substrate during and after the incubation period.

This can be explained by the fact that these eggs were non fertile or not fertilized. The snails (*Achatina fulica*) only nourished during the study with lettuce, grew very slowly in terms of the weight throughout the experiment.(ELMSLIE , 1986) mentions as many authors propose to bring permanently a source of carbonate of calcium in addition to other food as well in experimental breeding as in farm. (PEAKE, 1978) confirms the role of this mineral (calcium) in the fruitfulness and the production of eggs of the terrestrial gastropods. Calcium also intervenes in the formation of the shell which represents part of the live weight of snail. The starting average weight of the snails which was 63.66g reached its maximum (78 g only at the end of four months. (ADJIRI E, 1990) showed that the growth is faster at the *Archahatina* kind than at the *Achatina* kind. During January, strong heat led snails to be locked up in their shells (cover) while being nourished practically more what feels at weight level .The results of the mycological analysis revealed the presence of several cosmopolitan fungi: *Aspergillus Niger*, *Aspergillus terreus*, *Mucor sp.*, *Penicillium sp.*, *Phoma sp.* and *Trichoderma sp.* with a clear predominance of the *Aspergillus* kind. This predominance seems to be favored by moisture taking into account the fact that cotton retains water. (NOURZHANOV , 1987) had identified *Aspergillus terreus* on alive eggs of Moroccan locusts whereas this fungi seemed to be a saprophyte which colonizes dead eggs. *Aspergillus terreus* present on eggs of *Achatina fulica* could be regarded as a parasite of its eggs. The percentage of contaminated eggs on the whole is of 29.75 % rather weak because once the laying was detected, the eggs are recovered, cleaned and distributed at once on the substrate of incubation what does not allow the contamination of a great number of healthy eggs (white) by sick eggs (gray or yellow) disseminated in the laying. From a mycotoxicologic view, two among the five isolated kinds were potentially toxinogenes (Weindenborner, 2000). The stocks belonging to these kinds can synthesize various mycotoxins: aflatoxins which can be produced by *Aspergillus* and *Penicillium;* the ochratoxine A produced especially by *Penicillium*.

6 CONCLUSION

Absorbent cotton can be regarded as a good substrate of egg incubation of *Achatina fulica* because it is a good conservative of moisture and especially because of the fast and easy hatching of eggs on this substrate. The presence of fungi is not related to the nature of the substrate but rather with the presence of sick eggs. The permanent calcium contribution in addition to fresh lettuce would quickly have probably improved the snails' middle weight

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