Viability of earthworms’ egg-cocoon

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Abstract. The article suggests the methods of mass collection of earthworms and their cocoons as well as the identification of cocoons’ viability which stimulates the research of earthworm, in particular their reproductive capabilities. The absence of correlation between the size of cocoons and the quantity of warms, hatching from them, is found out. It is shown that the preliminary processing with glycerin or dehydration of earthworms’ cocoons significantly reduces the yield of viable individuals from frozen cocoons. The technology for transportation and keeping vermiculture in the earthworm-egg-cocoon stage was worked out. The technological scheming includes mass cocoon sampling, a cocoon lot preparation for keeping and transportation at room temperature for 30 days, and long-term keeping (about 5 months) at lower temperatures in wintertime.

1. Introduction

The cocoon stage is of great importance for the dispersal of earthworms [1], and it also increases the adaptability of certain type if oligochaetes to the transfer of unfavourable conditions [2]. Vermi-transformation provides a possibility of solving ecological problems of soil biocenosis [3] restoration by means of vermontransformation of household [4], agricultural [5] and industrial wastes [6]. As a result it causes the toxic substances neutralization, the vermicompost is formed [7], and the accumulating biomass of worms becomes a valuable protein for livestock, pharmaceuticals and the food industry. Vermiculture can also be used when the emergency situations arise for the remediation of territories contaminated with various pollutants [8], for example, petroleum products. There are interesting scientific studies on the acceleration of vermiculture growth [9,10].

However, the transportation of vermiculture presents a number of difficulties. Worms require special rooms, large volumes of substrate, stable environmental conditions (neutral pH, temperature 20-25 °C, humidity 80-85%, fertilizing, aeration) [11]. Therefore, to forward and store vermiculture, it is more convenient to use not the worms themselves, but their egg cocoons. The cocoons of the worms take considerably less space, the length of the adult worm is about 10 cm, and the cocoon is only 3-5
mm. There is no need for additional fertilization and such a bulky and heavy substrate as moist soil. Up to 20 young individuals can hatch from one cocoon. To use, store and transport vermiculture in the form of earthworm-egg-cocoon, a number of scientific and practical questions should be solved.

The aim of the research is to study the features of the earthworms’ physiology, which makes it possible to use their egg cocoons for effective settlement of vermiculture. In accordance with the goal of the work, the following tasks were determined to be solved:

1. to develop a technically simple and safe way of collecting earthworms and their cocoons;
2. to choose a technique for determining the viability of cocoons of earthworms;
3. to develop the methods for storing, including the long-term one, and the transporting of cocoons of earthworms;
4. to investigate the process and develop a technological scheme for storing and transporting vermiculture at the stage of earthworm-egg-cocoon.

2. Materials and methods

Red Californian worms (Eisenia fetida andrei Bouche, 1963) and their egg cocoons have been the object of the study. The worms were bred in cages with soddy-podzolic soil with maximum observance of all optimal parameters of vermicultivation [11]. The experiments involved sexually mature specimens 8-10 cm long.

For a quick selection of media for vermiculturing, the reaction of earthworm preference was used [12]. To do this, wooden containers were made with a size of 60x15x6 cm. With the help of easily retractable partitions, they were divided into the required number of chambers. The compartments were filled with the test substrates with a layer of about 5 cm. The partitions between the chambers were then removed. After that on the surface of the substrate the earthworms were evenly distributed (at the rate of 4 individuals per section). After a certain time (from 1 to 10 days) the partitions were returned to their original positions. That prevented the possible mixing of worms. Then the number of worms in each section was counted. The mixture where the largest number of copies crawled was taken for optimal number.

The earthworms fed with decoctions and extracts. Of the root crops, we took potato (Solanum tuberosum), carrot (Daucus sativus), and beet-root (Beta vulgaris var. Esculenta). Decoctions were cooked with pea (Pisum sativum), buckwheat (Fagopyrum esculentum), and rice (Oryza sativa) [13]. Water plant extracts [14] were prepared from leaves and seeds of nettle (Urtica dioica), clover (Trifolium pratense), chamomile (Matricaria matricarioides), dandelion (Taraxacum officinale), carnation (Dianthus versicolor), ribwort (Plantago major), and Canada water weed (Elodea canadensis). Decoctions involved 300 g of the product per one litter of settled dechlorinated pipe water boiled thereafter for 30-40 minutes. Water extracts were obtained by adding 50 g of thoroughly mashed plant biomass to a litter of water. Extracts and decoctions were filtered through gauze before feeding. For a food supplement, we also used dry yeast (Saccharomyces cerevisiae). Water pepper (Polygonum hydropiper) [15] growing along the bank of the Angara River was extracted in a similar way.

Cryoconservation of cocoons was performed in several stages:

A part of the cocoons was frozen without any preparation. Another part of the cocoons was put in a 10% glycerin-water solution for 24 hours before freezing. The third part of cocoons was one-day placed beforehand in sphagnum moss (Sphagnum sp.).

The cocoons were refrigerated and stored at –5-7 °C. For substrates, we used sphagnum moss and refined and deodorized sunflower oil. A part of the cocoons was kept in bottles without substrate.

1-5 months later, the cocoons were thawed at room temperature (about 20 °C) during 24 hours.

After thawing, the cocoons were put into settled water, and cryoprotector was thereby removed and water content in the cocoons was thereby normalized.

The state of earthworms was evaluated from biomass buildup, body dimensions, fecundity, and juvenile development.
The experimental designs included no less than six biological and four analytical replications. Statistical processing was performed with Statgraf® and Excel software. The conclusions were made with accurate forecast probability $P \geq 0.95$.

3. Results and discussions

Hand collection of a large number of earthworm cocoons is a time-consuming and insufficiently effective process [16]. In an effort to increase collecting capacity, we proposed sieving of substrate wherein the pubescent worms had been preliminary incubated. The average cocoon diameter is 3 mm. Because of this, the substrate was sieved through a 2-mm sieve.

At the first stage, we assessed the vermiculture substrate preference via preferential feeding of earthworms. To do this, we used soddy-podzolic soil, river sand, and gray clay. Consideration was being given to the reproduction rate of worms therein, and diameter and absorption ability of the substrate particles. Sand with a particle size of 1 mm appeared to fit all the criteria mentioned.

Warm feeding remnants prevent the separating of cocoons from substrate. Because of this, the feeding preference of the worms served as the basis for selection of liquid supplements providing optimum nutrition. The decoctions of root crops and grains, dry yeast, and plant extracts were tested then. Of all the supplements used, the decoction of buckwheat and fresh nettle extract appeared to be most effective. In these cases, the number of cocoons as obtained was 2-3.5 times more than the reference number.

The worms must be removed from the substrate as they may be subjected to mechanical damage in sieving. For this purpose, water pepper is a convenient choice. Within 30 minutes after the water extract of P. hydropiper had been added to the substrate, more than 90% of the worms crawled out to the surface. Of this, 60% worms entirely crawled out of the ground and for the rest of the worms it was partial crawling. Thereafter the upper substrate (5-10 cm) was collected together with the worms. The worms stayed at the surface and were non-mobile for the first 5 hours after the substrate processing. Then they again began to crawl into the substrate. The worms and the substrate could be used again even in a day. The experiments show that in a month after using water extract of P. hydropiper the size of the coprolites, reproduction rate and vitality of juveniles did not differ from the reference.

Some experimental results may be taken as an example. 200 g sand with a particle size of 1 mm was placed in a 15×10×5 cm Petri bowl. Twenty pubescent worms were put and incubated therein at optimal conditions. The worms were fed with a decoction of buckwheat and nettle extract once every three days. 20 ml of water pepper extract were added to the substrate after 10 days. The upper substrate collected after 30 minutes yielded about 94% of worms. The substrate was left to dry for 24 hours. Then the sand was sieved through a 2-mm sieve. Thus, 180.7 ± 12.6 cocoons were collected in 30 days.

Therefore, the proposed method includes the following steps:

- Worm cultivation in sand with a particle size of 1 mm.
- Feeding with decoctions of vegetables and grains or with plant extracts.
- Separation of worms from the substrate with water pepper extract.
- Sieving the substrate through a 2-mm sieve.

This method provides an efficient way to collect earthworm cocoons, decrease the mechanical damage to biological objects and significantly decrease the labor consumption.

For a long-term transportation or keeping over winter, it is desirable to use the cocoons with early-stage differentiated embryos to prevent vermiculture from being lost because of untimely hatch. Perhaps the simplest way to select cocoons of the same age is to consider their color, which is seen to change as the cocoons mature. The most suitable 5-10-day cocoons are of yellowish-milk color.

The sizes of red Californian worms vary greatly. It was suggested that the number of maturing individuals depends on the size of the cocoons. The presence of such correlation would certainly increase the number of worms transported by selecting larger cocoons.

A clear positive correlation was found between the length of the cocoons and their weight during the first stage of the study. Thus, the length of the cocoons varied from $2.5 \pm 0.2$ to $6.0 \pm 0.2$ mm, while
their weight varied from 7.9±0.7 to 22.9±0.5 mg, respectively. The thickness of cocoons is usually 2-4 mm, regardless of their length and weight. In the sample of 100 objects, In a sample of 100 objects there were cocoons 2.5 mm long - 6%, 3.0-18%, 3.5-10%, 4.0-26%, 4.5-12%, 5.0 - 22%, 5.5 - 4%, 6.0 - 2%.

As the cocoons mature, their weight has remained practically unchanged. Only from the 24th day there was noticed the reduction of weight of cocoons, which by the 30th day averaged 39.6% of the original, which is most likely due to the beginning of worms hatching.

There was no clear correlation between the size of the cocoons and the number of individuals hatched from them. Thus, most of all worms appeared from cocoons with a length of 5.0 mm - 31.5±2.5; then from cocoons with a length of 4.0 and 3.0 mm - 27.8±1.4 and 27.5±2.5, respectively; length 4.5 and 2.5 mm - 25.5±0.5 and 25.2±1.8; length 6.0 and 5.5 mm - 24.4±2.6 and 24.5±1.3.

Summarizing all the data, we can say that, depending on the length of the cocoons of red Californian worms (2.5-6.0 mm), their weight (9-23 mg) also varies accordingly. The most common cocoons are 4.0 mm long, they make up 26% of the total number. In the process of maturation, the weight of the cocoons practically does not change. It is impossible to judged by the size of the cocoon with the number of worms that hatch from them.

Besides, it is important to know the vitality of the cocoons before being used [17]. The quality of worm cocoons is traditionally determined from the quantity of hatching eggs [18]. However, it is a time-and labor-consuming method. That is why to estimate physiological condition of worm cocoons; we should have quicker and cheaper methods. The experiments showed the prospects for estimating vitality of worm cocoons out of their dehydrogenase activity with 2,3,5-triphenyltetrazolium chloride (TTC) [19]. Optimal conditions for manipulating cocoon color with TTC were selected. Distilled water was pored into test tubes, 5 ml in each. To each test tube, 0.5 ml TTC solution, 10 g/l, was added and ten 5-15-day old cocoons were put in it. Tight-closed test tubes were kept in a thermostat at 37 °C for 55 minutes. The degree of vitality of cocoons was determined 1-3 hours later by red color intensity. Dead cocoons remained uncolored. It is significant that this method yields results in just a few hours while hatching does it only in a month.

The selection of optimal substrates for storing and transportation worm cocoons involved testing of river sand (pH 6.6) and sphagnum (pH 7.2) and polytrichum mosses (Polytrichum commune) (pH 7.5). Soddy-podzolic (pH 7.7) soil was under control. A 30-day exposure of cocoons in these substrates showed that mosses have some advantages over soil and sand because they are much lighter in weight and of high water-holding capacity.

We suggest that polyethylene bags are the most suitable keeping and transportation containers for cocoons. The tests (Table 1) showed that using non-aerated bags and sphagnum substrate is the best way to keep embryos.

**Table 1.** Vitality of cocoons (from dehydrogenase activity and warm hatching number) kept in various substrates at room temperature.

| Substrate          | Number of alive cocoons, % of the reference soil | Average number of worms per cocoon |
|--------------------|-----------------------------------------------|-----------------------------------|
|                    | Time, days                                   |                                   |
|                    | 5    | 10   | 20   | 25   |                                   |
| Sphagnum           | 111±6.0 | 96±9.0 | 131±9.8 | 114±7.2 | 5.3±0.7                           |
| Sphagnum (aeration) | 78±6.8 | 58±6.0 | 71±10.0 | 57±6.0 | 0                                |
| Polytrichum        | 78±10.3 | 58±6.0 | 59±12.0 | 46±11.9 | 1.0±0.2                           |
| Polytrichum (aeration) | 72±5.6 | 39±7.5 | 0       | 0       | 0                                |
The results allowed recommending the following procedures for storing and transportation earthworm cocoons. 0.5-cm thick sphagnum layers (moistened with settled tap water to humidity 80-85%) and 5-10-day old worm cocoons are placed in non-aerated bags 40-10×8-15 cm. The number of cocoons in a layer may vary from 20 to 50 depending on a bag size. One such bag may contain respectively from 160 to 750 cocoons.

The cocoons may be kept in such bags at room temperature for about 30 days until complete maturity. Under these conditions, newly hatched larvae lived for as much as 10 days. No evidence was found for growth and maturation impairments in these worms.

Long-term frozen storage of worm cocoons [20] showed that cryoprotecting with glycerin or dehydration results in death the cocoons or hatched worms. The presence of substrate (sphagnum moss or sunflower oil) caused, on average, 34% decrease in worm yield and 23% decrease in worm vitality.

Therefore, we would recommend worm cocoons freezing without cryoprotection and substrate. Such conditions provided the maximum yield in worms (about 80% of the reference) at 100% hatching survival. Besides, most of the worms remained alive and able to feed normally and to reproduce 90 days after this procedure.

Hence, the technological scheming of keeping and transportation the vermiculture in the worm-egg cocoon stage (Table 2) includes mass cocoon sampling, a cocoon lot preparation for keeping and transportation at room or lower temperature for 30-150 days.

Table 2. Keeping and transporting vermicomposting species in the worm egg cocoon stage.

| Stage                           | Processes                                      |
|---------------------------------|------------------------------------------------|
| Obtaining cocoons               | Cultivating worms in sand with a particle size of 1 mm |
|                                 | Feeding with decoctions and plant extracts      |
| Collecting cocoons              | Removing worms from the substrate with water pepper |
| Preparing cocoons for keeping or transportation | Sieving substrate through a 2-mm sieve |
| Keeping or transportation of cocoons | Selecting cocoons of the same age by color |
| Substrate colonization by vermiculture | Determining vitality with dehydrogenase |
|                                 | For 30 days at room temperature               |
|                                 | Long-term keeping at 5-7 °C                   |
|                                 | In the egg cocoon stage                       |
|                                 | In the adult stage                            |

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