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MASS PRODUCTION OF THE BENEFICIAL NEMATODE STEINERNEMA CARPOCAPSAE UTILIZING A FED-BATCH CULTURING PROCESS

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A B S T R A C T

The present study deals with the batch and fed-batch mass production of *Steinernema carpocapsae*. *S. carpocapsae* is an entomoparasitic nematode that is used as a biological control agent of soil-borne crop insect pests. The ability and efficiency of fed-batch culture process was successful through the utilization of the nematode's bacterial symbiont *Xenorhabdus nematophila*. Results from the fed-batch process were compared to those obtain from the standard batch process. The fed-batch process successively improved the mass production process of *S. carpocapsae* employing liquid medium technology. Within the first week of the fed-batch process (day six), the nematode density obtained was 202,000 nematodes mL^{-1}; whereas on day six, batch culture mode resulted in a nematode density of 23,000 nematodes mL^{-1}. The fed-batch process was superior to that of batch production with a yield approximately 8.8-fold higher. In fed-batch process, the nematode yield was improved 88.6 % higher within a short amount of time compared to the batch process. Fed-batch seems to make the process more efficient and possibly economically viable.

Keywords: *Steinernema carpocapsae*, *Xenorhabdus nematophila*, fed-batch fermentation, beneficial nematodes.

INTRODUCTION

Entomoparasitic nematodes (EPNs) have arisen as excellent biological controlling agents of crop insect pests. Mainly, due to many of their biological traits that include a broad insect host range, transmission of highly virulent symbiotic bacteria into the insect, safety towards non-target organisms (plants, livestock, humans, etc) and their high efficacy in the environment (Mahar *et al*., 2004). EPNs are now appearing for widespread usage because they minimize the use of chemical insecticides that is responsible for soil and water pollution. In the past, serious health risks to both humans and animals have occurred due to chemical insecticide usage (Inman and Holmes, 2012a). Furthermore, the number of insects that are devastating crops is continuing to increase due to the increase of insect resistance towards these chemical insecticides. The use of EPNs is very effective towards insects due to the chance of insects becoming EPN resistant is relatively low. Because of their attractive traits, EPNs are now being explored as a more environmentally-friendly substitute for chemical insecticide (Pfeifer and Grigliatti, 1996). Over the past two decades, commercialization of these nematodes has increased; however, their mass production *in vitro* is rather challenging as their biology is not fully understood. Furthermore, the utilization of solid media used in culturing technologies has been extremely successful; however, this method is not economically feasible due to the high cost of materials and supplies (Gaugler, 1997). On the other hand, researchers suggest that the development of liquid culture *in vitro* technologies would be economically feasible because a higher nematode density can be achieved utilizing simple complex media (Friedman, 1990; Inman and Holmes, 2012a). The infective juvenile (IJ) stages of *Steinernema* and *Heterorhabditis* nematodes have been dynamically used for regulating primarily soil-dwelling larval stages of various insect species. To control insects, EPNs work by transmitting deadly symbiotic bacteria into the insect host (Ciche *et al*., 2006). These entomopathogenic bacteria belong to the two genera *Xenorhabdus* and *Photorhabdus*. *Xenorhabdus* bacteria are symbiotically associated with *Steinernema*; while *Photorhabdus* bacteria are connected with

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Heterorhabditis. Both bacterial genera undergo a biological phenomenon that is commonly referred to as *phase variation*. This process causes bacteria to shift their metabolic activities from an unstable, pathogenic state (phase I) to a stable, less pathogenic form (phase II) when environmental conditions become unfavorable (Forst and Nealson, 1996). Many reports suggest that prolonged subculture under stressful conditions increases the likelihood and production of stable secondary phase cultures. It is important to note that mechanisms that trigger phase variation are yet to be identified (Akhurst *et al*., 1992; Crawford *et al*., 2010; Leclerc and Boemare, 1991; Wang and Dowds, 1993). Phase variation is a substantial factor associated with the delicate symbiosis between bacteria and nematode. Culturing must be closely monitored during the course of mass production due to phase transitioning (Forst and Clarke, 2002; Inman and Holmes, 2012a). It should be appreciated and acknowledged that the phase I state of bacterial symbionts secrete a battery of toxins and enzymes that kill and bioconvert the insect host for nutrition for the symbiotic partners. Furthermore, these unstable phase I variants also produce a wide range of antimicrobials that prevent other organisms from invading the insect cadaver, and by doing so; the bacterial symbionts produce an ideal breeding ground for its nematode partner *in vivo* or *in vitro* (Forst and Clarke, 2002; Inman and Holmes, 2012a).

Xenorhabdus is transported within the intestines of the infective juvenile stage (IJ) and is released into the hemocoel of the insect host upon nematode entrance. Within the cadaver, the bacterium proliferates and kills the insect within 24-48 hours with the mutual action of nematodes. In the insect hemolymph, bacteria will secrete “food signals” that induces the IJs to recover and continue the nematode life cycle (Akhurst, 1980; Golden and Riddle, 1984; Hirao and Ehlers, 2009). After bacterial signaling, infective juveniles lose their protective sheath and develop into feeding-stage 3 juveniles (J3). The J3 nematodes feed upon the proliferated bacteria and develop into the J4 stage and then to amphimictic adults. Due to the current status of food sources (bacterial density), the adult nematodes undergo reproduction. Under nutrient rich conditions, the adults undertake sexual reproduction (male and female) that spawn fertilized eggs. From microscopic observations, reproductive females can deposit up to 25-30 eggs (data not shown). Under nutrient depletion and/or adverse environmental conditions, adult nematodes fail to copulate and thus the female will turn to hermaphroditic reproduction. Observations of female hermaphrodites will produce 10-15 live IJ (data not shown) in a process known as *endotokia matricida* (Johnigk and Ehlers, 1999a, b). Endotokia matricida is the process where the hermaphroditic female gives rise to live young (J1) from eggs that will feed upon the maternal parent from within. As nutrients within the hermaphrodite decline, the young juveniles will collect phase I cells of the bacterial symbiont, convert to IJ status and exit the adult nematode. Within nutrient depleted insect hosts, the young J2 stage of both *Steinernema* spp. and *Heterorhabditis* spp., which are spawned from fertilized eggs, will develop into the infective third juvenile stage (IJ) (Strauch and Ehlers, 2000).

Production of nematodes is performed utilizing *in vitro* liquid technology in industrial-scale bioreactors and by doing so has greatly improved yield and quality while reducing costs. If perfected, liquid technologies can greatly increase IJ yields that are highly active and infectious within a shorter time period (Ehlers, 2001; Hirao *et al*., 2009). Successes in this technology usually involve the conditioning of the nematode production media by cultivating the bacterial symbiont within it prior to nematode inoculation (Ehlers *et al*., 1998). Nematode recovery in liquid culture can vary considerably based upon many factors such as: bacterial phase variant, media formulation, bacterial density, and/or the concentration of the produced “food signal” (Aumann and Ehlers, 2001; Inman and Holmes, 2012a). The highest recovery of IJs was achieved when culture filtrates of *P. luminescens* were obtained while the symbiont was in the late logarithmic phase (Strauch and Ehlers, 2000). According to Friedman (1990), recovery is crucial to accomplish an economically-friendly liquid culture process.

In glucose fed-batch cultures, the stability of the *P. luminescens* primary form was sustained for a longer time frame within stationary growth. Furthermore from the study, phase variation was avoided and a high cellular density was obtained through the addition of glucose. The high bacterial density is crucial for mass production as the bacterial symbiont is the main food source of the nematode (Jeffke *et al*, 2000). Based on the preceding information, the present study describes the propagation of the EPN *Steinernema carpocapsae* in submerged culture with its bacterial symbiont, *Xenorhabdus nematophila* utilizing a modern

fermentation system. During mass production, attention was focused onto two aspects: 1) use of modified LB media having rich sources of protein and lipid and 2) comparison of batch & fed-batch production processes. It is to the authors' knowledge that there are no reports regarding fed-batch production of *Steinernema carpocapsae*.

MATERIAL AND METHODS

Nematode and Bacterial Isolation: *Steinernema carpocapsae* nematodes obtained from ARBICO Organics (Tucson, AZ USA) were used throughout this study. Nematode sanitization and isolation of the bacterial symbiont *Xenorhabdus nematophila* were performed by the method of Inman and Holmes (2012b). Deceased insect larvae were surface sterilized by plunging them into 70% ethanol for 3-5 seconds and air-dried. Cadavers were aseptically dissected with a scalpel and a loop full of infected hemolymph was streaked on to media (NA and NBTA, see below) and incubated at 28 ºC (Inman and Holmes, 2012b).

Culture Media: Expressed in g L^{-1}:

NB (*Nutrient Broth*) – 5 g peptone, 3 g beef extract or yeast extract, 5 g NaCl and 15 g agar in the case of NA (nutrient agar); pH: 7.3

LB (*Luria Broth*) – 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar in case of LB agar; pH 7.3

NBTA – 8.0 g nutrient agar; 25 mg bromothymol blue; 40 mg 2,3,5 triphenyl-tetrazoliumchloride (TTC)

Production Media (*proprietary formulation*) – basic nutrients; animal tissue; lipid; physiological pH. Media also used for bacterial acclimation.

Bacterial culture and growth conditions: Bacterial colonies obtained from infected hemolymph were subcultured onto nutrient and LB agar until colonies of uniform size and morphology were obtained. For confirmation of phase variant, *X. nematophila* was cultured onto NBTA media. Phase I adsorbs bromothymol blue and reduces TTC, producing dark blue colonies with a red core, whereas cells of phase II do not adsorb bromothymol blue but reduce TTC and produced maroon colored colonies (Akhurst, 1980, Inman and Holmes, 2012a).

Bacterial stock cultures were established in 3 mL of LB and incubated at 28ºC for 24 hours agitated at 150 rpm. For bacterial acclimation, 50 mL of production medium was inoculated with *X. nematophila* stock culture (5%), incubated at 28ºC and agitated at 150 rpm. To ensure acclimation, cultures were transferred to production medium every other day.

Nematode mass production: For fed-batch culturing, the production medium (3 L) was conditioned with the bacterial symbiont in a Sartorius stedim Biostat B plus 10 L bioreactor. The culture was incubated at 28ºC; agitated at 400 rpm while pH and oxygen saturation was maintained at 7.30 and 70%, respectively. Infective juveniles were surface sanitized using 0.125% hyamine and rinsed with sterile tap water. After bacterial growth reached stationary phase (24 h), 500 mL of the bacterial symbiont was removed and replaced with 500 mL of fresh medium containing ~ 4,000 sanitized IJs mL^{-1} to initiate the production process. Prior to nematode inoculation, agitation was reduced to 100 rpm and DO increased to 100%.

After every 24 hours, 500 mL of the culture was aseptically withdrawn and centrifuged for 5 minutes at 500 rpm to pellet the collected nematodes. The supernatant was discarded and the pellets were resuspended and transferred to 500 mL of fresh production media containing 100 µg mL^{-1} of ampicillin and added to the reactor. For batch production, all process parameters and media remained the same; however, spent media was not removed and nothing was added to the reactor.

Determination of nematode yield & recovery: To determine the nematode concentrations over time, samples from the removed fed-batch volumes were diluted to 10 to 1,000 times with sterile tap water. Triplicates of 0.1 mL samples were used for microscopic counting of total nematodes utilizing brightfield microscopy at 40X magnification with the resultant count multiplied by the respective dilution factor. To observe nematode development, a magnification of 100X was used.

Harvesting protocol: After six days of production, high concentrations of IJs were observed in the production media. On day seven, the culturing media was collected and centrifuged at low speed to pellet adult nematodes. Secondly, the supernatant was transferred and centrifuged at medium speed to pellet juvenile nematodes. The pellet containing adults were washed several times with sterile water and reincorporated into the production medium. The pellet consisting of juveniles were also rinsed in sterile water; however, some of the juveniles were destined for packaging and the remaining juveniles were used to inoculate another pre-conditioned production medium. This general protocol can be seen in Figure 1.

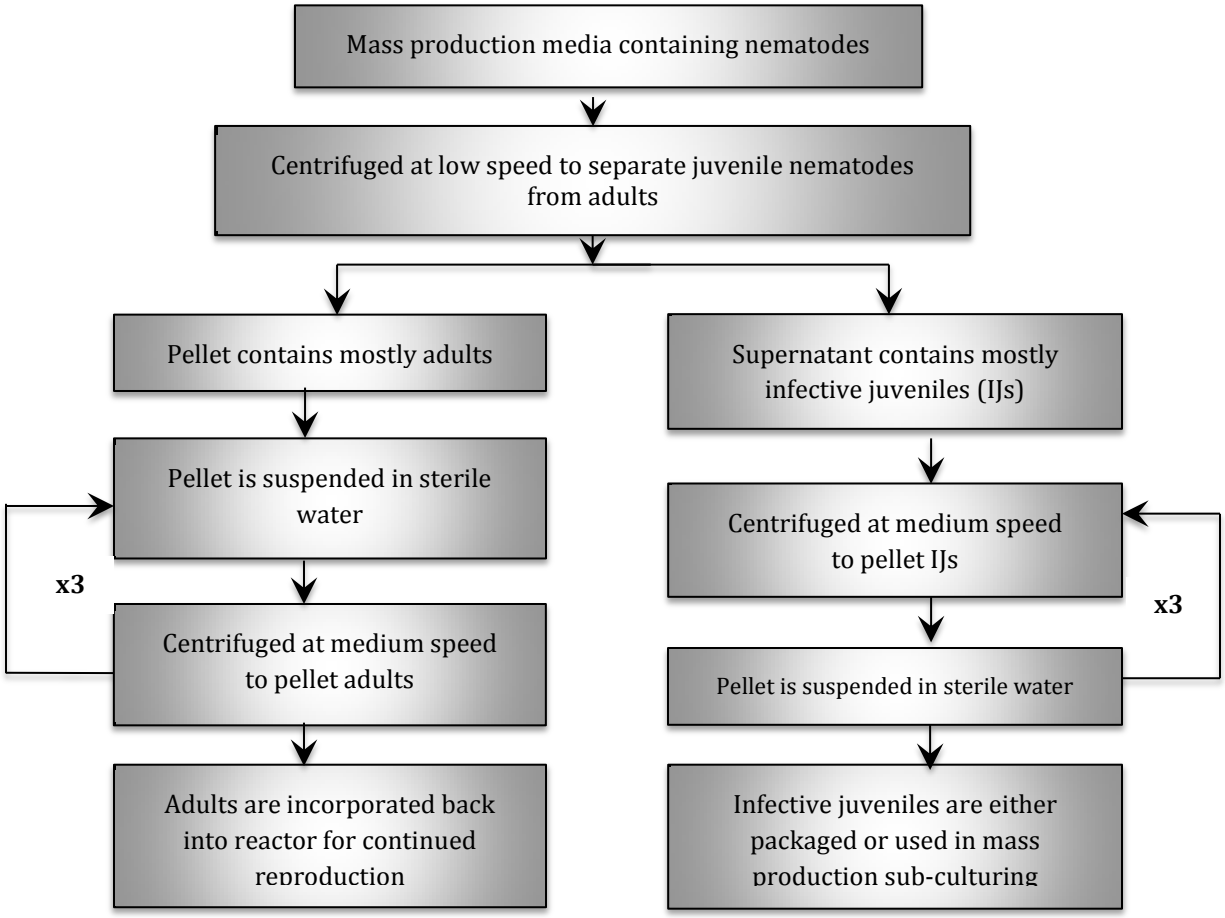

Figure 1: Schematic of general harvesting protocol

RESULTS AND DISCUSSION

Growth of *Xenorhabdus nematophila*: It is necessary to make attempts to acclimatize *X. nematophila* to the LBC production medium. Figure 2 presents microphotographs of Gram-stained *X. nematophila* cultured in LBC just at the time of inoculation (a) and 48 hours post-inoculation (b). 'It is obvious through these microphotographs that the bacterium took easily to the production medium as the cells appear to be extremely "healthy;" however, the increase of bacterial density was not determined as the complexity of the medium prohibited optical measurements. During the study, chicken was used to provide rich sources of protein and lipid that seems to favor bacterial and nematode growth.

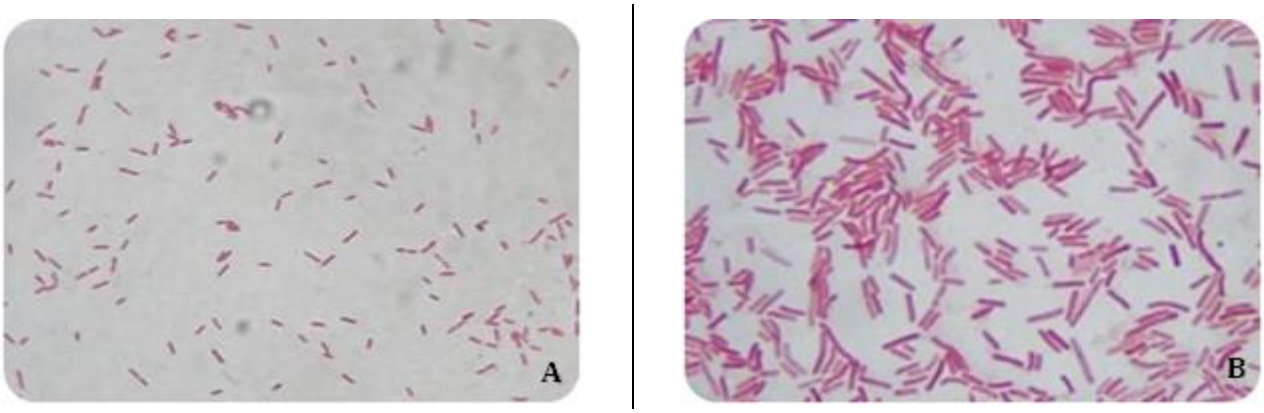

Figure 2: Light microphotographs (1,000X) of *Xenorhabdus nematophila* growing in LBC. Culture samples at: t = 0 h (a) and t = 48 h (b). Upon gross cellular morphology, it is apparent that *X. nematophila* can be easily cultivated in production media.

Mass production and Development of *Steinernema carpocapsae*: Figure 3 depicts the increase of nematode densities as a function of time and the fermentation mode used for mass production. During the first 3 days of both batch processes, nematode density did not change (~4,000 IJs ml^{-1}). However, in the fed-batch process, IJs recovered and rapidly developed into J4s and egg-producing adults. By the end of day 4, J1 and J2 juveniles were observed. The most dynamic growth seen in the nematode population of the fed-batch process occurred around day 5. On the contrary, recovering of IJs in batch mode was much more slowly and adults were finally observed on day 5. Maximum nematode concentrations after six days post-nematode inoculation for both fed-batch and batch processes were 202,000 IJs mL^{-1} and 23,000 IJs mL^{-1}, respectively. The difference in the obtained densities is mainly due to nematode recovery and the increasing number of reproductive females (Hirao and Ehlers, 2010). Furthermore, the fed-batch process ensured that the bacterial symbiont remained healthy within the reactor to support an increased recovery rate (data not shown) resulting in higher yields of reproductive females. Various stages of *S. carpocapsae* development are shown in Figure 4.

In the present study, fed-batch produced a nematode yield that is 88.6 % more efficient than the batch process. Gil *et al*. (2002) had reported a 32% higher yield of *Heterorhabditis bacteriophora* in fed-batch when compared to a batch process containing glucose and lipid. Jeffke *et al*. (2000) studied a glucose fed-batch process with the bacterial symbiont *P. luminescens* where the authors were able to obtain a high bacterial density. Furthermore, the authors did not detect any phase shift during the culturing period or during an extended stationary growth period. Another advantage of culturing bacterial symbionts to high densities using glucose supplementation is the increase in concentrations of secreted antimicrobial compounds that could be exploited to minimize reactor contamination (Hu and Webster, 2000).

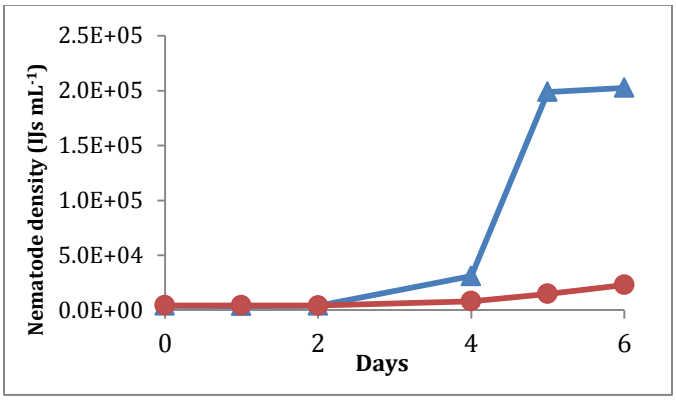

Figure 3: Increase in *Steinernema carpocapsae* densities as related to time and production process. At the end of the production period (6 days): batch process (●) was able to produce a final nematode density of 23,000 IJs mL-1 while fed-batch (▲) was able to generate a final concentration of 202,000 IJs mL-1.

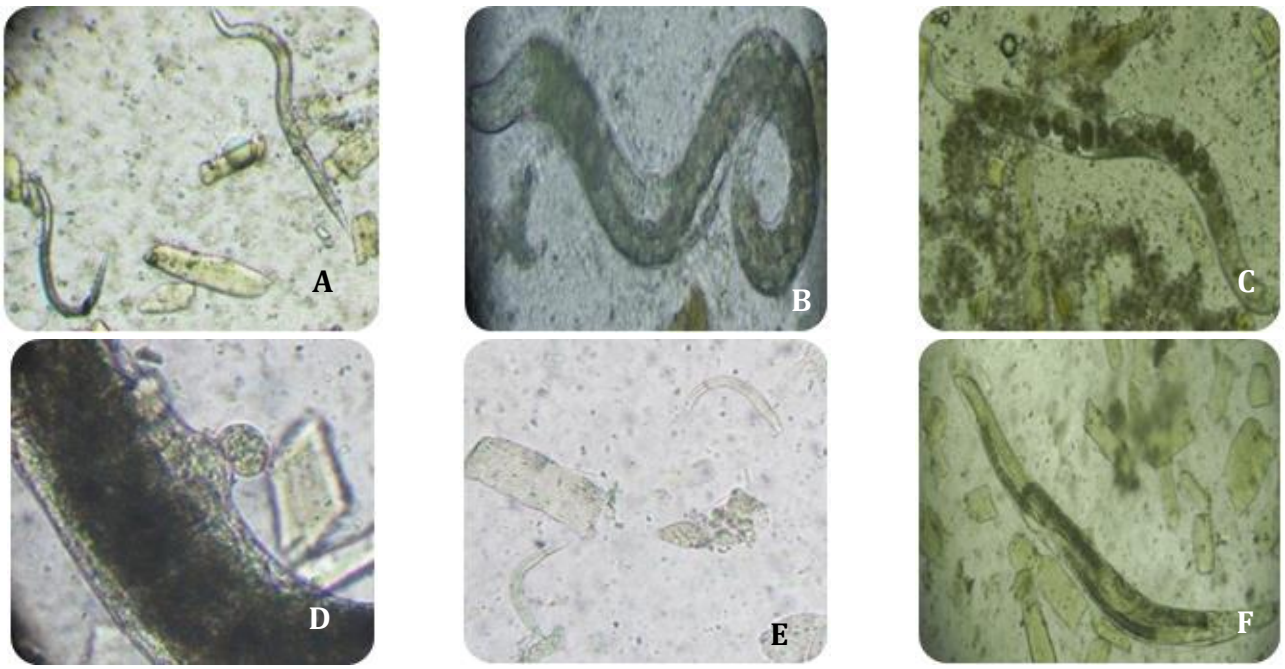

Figure 4: Light microphotographs of development stages of *Steinernema carpocapsae* during development in LBC medium; (A) IJ3; (B) young hermaphrodite; (C) adult; (D) egg-laying; (E) J1; (F) *endotokia matricida.*

CONCLUSION

The present study provides valuable guidance on implementing a fed-batch process for mass production of *S. carpocapsae*. Fed-batch enhanced IJ recovery by maintaining *X. nematophila* in the phase I state and as a consequence improved IJ production within a shorter period when compared to standard batch production. Furthermore, the production of high concentrations of IJs utilizing fed-batch occurs during the development of the first nematode generation (Wang and Bedding, 1996). According to Ehlers *et al*. (1998), fed-batch production of *Heterorhabditis bacteriophora* generates higher densities within the first generation when compared to batch production in which densities are highest after the second generation. To the best of the authors' knowledge, this is the first report on utilization of a fed-batch process for the mass production of *Steinernema carpocapsae*.

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