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Effects of ecdysteroidal extract of *Spinacia oleracea* on demographic parameters of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae)

Bibi Zahra Sahaf and Saeid Moharramipour*

Department of Entomology, Faculty of Agriculture, Tarbiat Modares University, P. O. Box: 14115-3361, Tehran, Iran.

Abstract: Plant extracts, such as phytoecdysteroids, are currently studied because of the possibility of their use in plant protection. Phytoecdysteroids are insect steroid hormone analogues, and they are believed to deter invertebrates from plants, either by acting as antifeedants or by being toxic through hormonal disruption upon ingestion. We describe here the effects of extract from *Spinacia oleracea* L. (Chenopodiaceae), a plant producing phytoecdysteroids, on the demographic parameters of *Plutella xylostella* L. (Lepidoptera: Plutellidae). Spinach is one of the very few crop plants which produce large amounts of phytoecdysteroids. Ecdysteroidal extracts of leaves from this plant were incorporated into food given to third instar larvae for two days. Then the larvae were reared on untreated leaves. The eggs from the emerging adults were picked up for demographic experiments. The rearing of the newly hatched larvae was continued individually on untreated leaves. All experiments were performed at 25 ± 1 ºC, 65 ± 5% RH and a photoperiod of 16:8 (L: D) h in a growth chamber. Data analysis demonstrated that the fecundity of the females was strongly affected by ecdysteroidal extract. Values of intrinsic rate of increase and net reproduction rate decreased significantly as concentration of the extract increased. However, doubling time increased significantly as concentration of the extract increased. The present study demonstrated that the ecdysteroidal components of spinach are effective on the demographic parameters of *P. xylostella*. Therefore, this extract may be a potential protectant as botanical alternative agent.

Keywords: Diamondback moth, *Plutella xylostella*; phytoecdysteroids, demography; biological parameters; *Spinacia oleracea*

Introduction

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) is one of the most destructive insect pests of brassicaceous crops in the world. The global importance of DBM is reflected in estimated control costs of approximately US$ 1 billion per year (Talekar and Shelton, 1993; Verkerk and Wright, 1996). Many species of brassicaceous crops are cultivated as vegetables and oil seed crops. Some weed species of crucifers are fed by DBM in absence of their favored crop hosts and play important link in maintaining DBM populations (Talekar and Shelton, 1993; Begum *et al.*, 1996). Even though insecticides remain as the first defense method against the DBM, the evolution of

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* **Corresponding author**, e-mail: moharami@modares.ac.ir
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resistance to pesticides has become a major problem (Shelton *et al*., 1991).

Plant extracts like phytoecdysteroids have been a subject of research in an effort to develop alternatives to the conventional insecticides. Phytoecdysteroids occur in a wide range of plant species, where they contribute to the deterrence of phytophagous invertebrates. Some of the plants from the families of Chenopodiaceae (Dinan, 1995), Caryophyllaceae (Zibareva *et al*., 2003) and Solanaceae (Savchenko *et al*., 2000) have been found to contain phytoecdysteroids. Most crop species do not contain detectable levels of phytoecdysteroids. Spinach (*Spinacia oleracea* L.) is one of the few cultivated phytoecdysteroid-accumulating species (Chenopodiaceae) (Dinan, 1995), and therefore represents an interesting model system for a detailed analysis of extract that has phytoecdysteroid effects on insects.

Population parameters are important to measure population growth capacity of a species under specified conditions. These parameters are also used as indices of population growth rates responding to selected conditions (Southwood and Henderson, 2000). The construction of life tables is appropriate to study the dynamics related to the population growth potential, also called demographic parameters (Carey, 1993, 2001; Southwood and Henderson, 2000). The intrinsic rate of natural increase (r_m) is a key demographic parameter useful to predict the population growth potential of an animal under a set of given conditions (Andrewartha and Birch, 1954; Ricklefs and Miller, 2000). A number of extrinsic and intrinsic factors such as certain glucosinolates, cardenolides, plant volatiles, waxes and leaf morphology have been shown to affect the r_m value and related demographic parameters (Gilbert and Raworth, 1996; Lee and Elliott, 1998; Syed and Abro, 2003; Sarfraz *et al*., 2006). The current study is evaluating the effect of ecdysteroidal extract of *S. oleracea* on the life table, population growth and reproduction parameters of the DBM. Study of life table parameters of DBM as affected by this extract is required in order to develop an alternative to the conventional insecticides.

Materials and Methods

Rearing of *P. xylostella*

The initial population of DBM was collected from *Brassica napus* L. in the fields of the horticultural investigation centre of Tehran University, Karaj, Iran during October 2009. The stock culture of *P. xylostella* was initiated on different potted host plants and maintained at 25 ± 1 ºC, 65 ± 5% RH and a photoperiod of 16:8 (L:D) hours in a growth chamber.

The host plant, canola, *B. napus* (cultivar Opera) was planted in pots in a greenhouse. Host plant seeds were sown in suitable soil and compost mixture in flats. After five weeks when plants were at 6 to 8 leaf stage each seedling was transplanted in 20 cm diameter plastic pot. When host plants had 10-12 leaves they were used for experiments.

In order to obtain the same aged eggs of DBM, host plant leaves were placed inside oviposition cages containing 8-10 pairs of both sexes of DBM which were identified by visible differences in their external genitalia (Liu and Tabashnik 1997). The oviposition cages were transparent, cubic Plexiglass containers (15 × 8 × 5 cm), with a fine nylon mesh installed on the top-side. After 12 h, the host plant leaf was removed from the cage. A small cotton-wool which had been soaked in 10% honey solution was placed in each oviposition cage as a source of carbohydrate for adults.

Plant material

Leaves of *S. oleracea* cultivar Barg-Pahne Varamin were harvested in August 2009 from the field of Tarbiat Modares University, Tehran, Iran.

Extraction of plant material

The leaves (50 g) were ground and sonicated with methanol 70% (3 × 250) at 55 ºC for 3 h. The aqueous methanol phases were combined and the solvent was evaporated under vacuum

at 55 ºC and 120 rpm in 3 h (Heidolph, Germany). The residue was stored at minus 24 ºC. The amount of the residue was 6 ml. The methanolic extract was fractionated on Chromabond C18ec cartridge from Macherey-Nagel Company. At first the cartridge was washed with 3 ml of methanol and then 6 ml water to activate. Then extract was dissolved in 10% methanol in water and loaded on to the cartridge. Then the cartridge was eluted with 3 ml from each of 25:75, 60: 40 and 100: 0 (by volume) methanol: water, respectively. The purified extract was kept in refrigerator at minus 24 °C until the start of experiments.

Survivorship, mortality and fecundity

All experiments were carried out in a growth chamber set at 25 ± 1 °C, 65 ± 5% RH and 16: 8 (L: D) h. The purified extract of spinach was used in the experiments. The tested concentrations against DBM were 12.75%, 16.96% and 20.84% purified extract (LC_{10}, LC_{25} and LC_{50} of third instar larvae) that contained 2.1, 2.8 and 3.4 µg/ml 20-hydroxyecdysone, respectively. The leaves of the *B. napus* were soaked in the purified extracts for 15 seconds. Control leaves were soaked in methanol. Treated leaves were left in the Petri dishes (9 cm diameters) after drying the solvent. The third instar larvae were fed on these leaves for 48 h. After that, the larvae were transferred onto untreated leaves and maintained up to adult stages. The adults that emerged from third instar larvae were transferred to the oviposition cage for 24 h. After oviposition the eggs of DBM were picked up from the surface of the host plant leaves using a small brush and placed individually in Petri dishes (8 × 1.5 cm) on a leaf of host plant. The petioles of detached leaves were inserted in water-soaked cotton-wool to preserve freshness. Lids of petri dishes were covered with fine nylon mesh for aeration. At least 70 eggs of DBM were used to collect data for each concentration. The eggs were checked daily and their developmental stages were recorded. The larvae were fed on fresh leaves and they were replaced every day awaiting the larvae death or their reaching the prepupal stage. The presence of exuviae was used to discriminate the larval instars. The regular checking of petri dishes continued, until the entire individuals became adults.

The survival rate and development time were recorded for all immature stages. The adults sex ratio reared on the rapeseed Opera cultivar was also determined. A fertility life table was constructed according to Birch (1948) and Carey (1993, 2001) based on the data obtained from the incubation of the eggs, duration of nymphal instars, age specific mortality/survivorship and fecundity.

Population growth parameters

For each concentration, 25 pairs of newly emerged adults (n = 25 replications) were transferred to transparent plastic cages (15 × 8 × 5 cm). The host plant leaves were replaced with fresh ones daily. Then, number of eggs laid by each female was recorded until death of the entire adult females.

Data analyses

Age-specific survival rates (l_x) and average number of female offspring (m_x) for each age interval (x) were used to construct age-specific fertility life tables. Using survivorship and fertility schedules, the demographic parameters of DBM including net reproduction rate (R_0), intrinsic rate of increase (r_m), finite rate of increase (λ), mean generation time (T), doubling time (DT) and life expectancy (e_x) were calculated. All terminology and formulae for computing demographic parameters are consistent with Carey (1993).

The jackknife method was used to estimate the variance for r_m and other reproduction and population parameters (Meyer *et al.*, 1986). The technique is based on the repeated recalculation of the required estimator and missing out each sample in turn (Maia *et al*., 2000). The jackknife pseudo-values for each rapeseed cultivar were subjected to the analysis of variance (ANOVA).

The same procedures were used for the other parameters *Ro*, λ, *T* and *DT*. If significant differences were detected, multiple

comparisons were made using the LSD test ($P < 0.05$) (Maia *et al*., 2000). Statistical analysis for reproductive parameters was carried out using SPSS statistical software (SPSS, 2004). Data were checked for normality test prior to analyses and the multiple comparisons were made using the Tukey's HSD test ($P < 0.05$).

Results

Survivorship, mortality and fecundity

The l_x of DBM on different concentrations of ecdysteroidal extract is given in Fig. 1. The entire individuals in the cohort of DBM reared on control and concentrations of 12.75%, 16.96% and 20.84% died at the ages of 20, 23, 20 and 19 days, respectively. The preimaginal mortality of DBM was 0, 5.64, 7.15 and 13.34% in control, and at concentrations of 12.6, 16.0 and 20.9% respectively. The m_x of DBM on different concentrations of the ecdysteroidal extract is shown in Fig. 1. The oviposition period of females was initiated at the age between 12 and 14 days depending on concentrations of the extract. The peak of female oviposition was observed at the age between 15 and 18 days (Fig. 1). In the same order, the e_x of DBM at the age of newly laid eggs (one-day-old) was 18.42, 19.37, 16.78 and 14.20 days, and at the age of adult emergence was 7.42, 9.90, 6.63 and 5.02 days in the control and the respective concentrations, respectively (Fig. 1).

The gross fecundity rate (mean number of eggs/female/generation) was significantly different for different concentrations of the ecdysteroidal extract ($F = 3531.55$; df = 3, 70; $P < 0.0001$). The descending order of gross fecundity rate was estimated on the concentrations examined. The mean number of eggs per female per generation was the highest in control. The net fecundity rates indicated significant differences among different concentrations of the ecdysteroidal extract ($F = 22117.06$; df = 3, 70; $P < 0.0001$). The gross reproductive rate was significantly different for the ecdysteroidal extracts ($F = 25394.14$; df = 3, 70; $P < 0.0001$)

The sex ratio of DBM was affected by different concentrations of the ecdysteroidal extract too. The sex ratio of progeny was more female-biased on all concentrations (Table 1)

Population growth parameters

The *in vitro* population growth parameters of DBM fed on different concentrations of the ecdysteroidal extract are shown in Table 2. There were significant differences among the *Ro* on control and different concentrations of the ecdysteroidal extract. The highest amount of *Ro* was observed in control. The r_m was also found to be significantly different in control. The r_m values ranged from 0.311 to 0.245 day^{-1} and the highest r_m value was recorded for control. Consequently, DBM is rather susceptible to this ecdysteroidal extract. The highest value of λ was obtained on control and was significantly different from those of the different concentrations of the extract. The *DT* was also found to significantly differ within the control and concentrations examined (Table 2). The *T* also significantly differed within control and treatments. The DBM had longer generation time in concentration of 12.6% than in the others (Table 2).

Discussion

The presence of adequate amounts of phytoecdysteroids in *S. oleracea* enabled us to rear larvae of an important pest, *P. xylostella*, on diets supplemented with ecdysteroidal extract. The data presented in this report clearly show the susceptibility of *P. xylostella* to the extract. When this extract was incorporated into the food of larvae, it caused numerous disruptions in life table, population growth and reproductive parameters. Using HPLC system (column Develosil, 250 mm long, 4.6 mm i.d.), the presence of 20-hydroxyecdysone was proved in the extract of *S. oleracea* leaves. The content of 20-hydroxyecdysone was 97.338 µg/50 g fresh weight of the leaves (Sahaf and Moharramipour, Unpublished data).

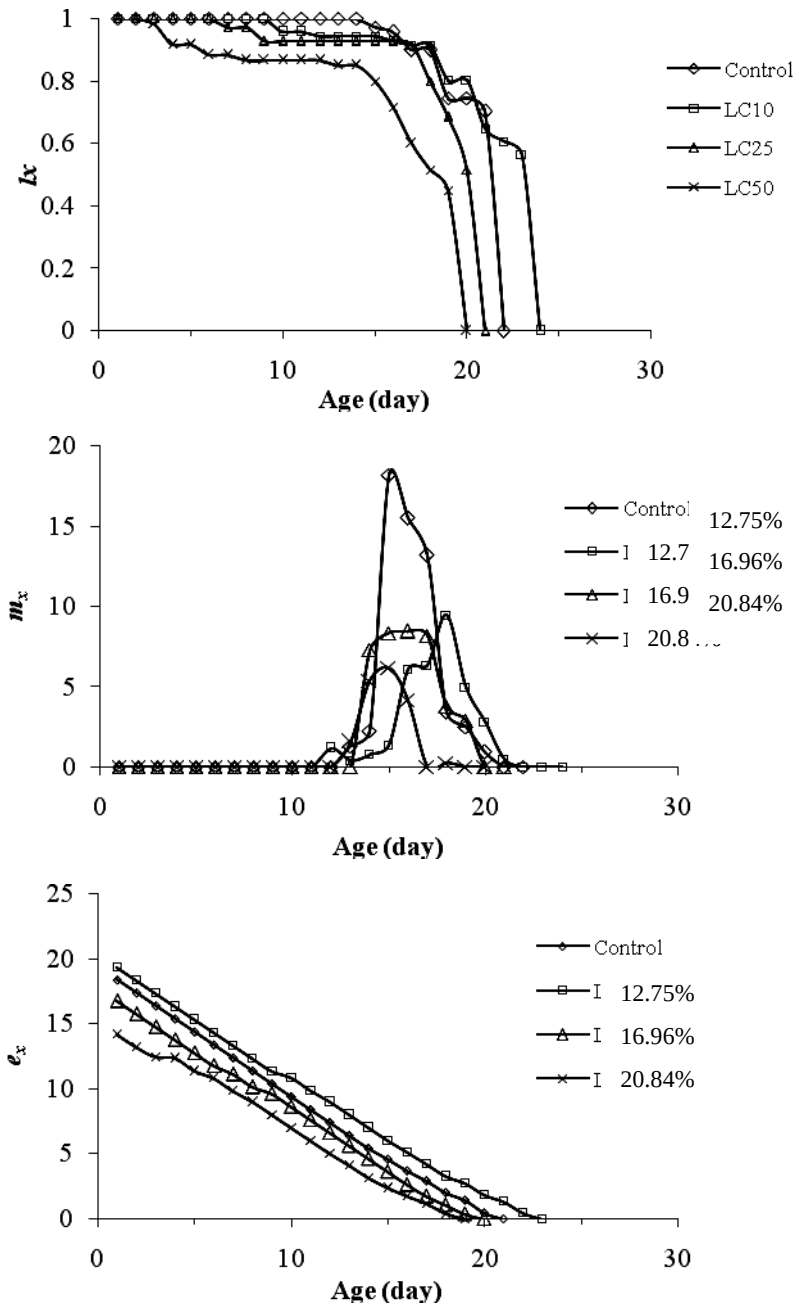

Figure 1 Age-specific survivorship (l_x), life expectancy (e_x) and age-specific fecundity (m_x) of *Plutella xylostella* on leaves treated with *Spinacia oleracea* ecdysteroidal extract.

The toxicity of ecdysteroidal extract on *P. xylostella*, appeared with the effects on intrinsic rate of increase and disruption of demographic parameters. Similar effects have been observed in other Lepidopteran species after ingestion of different phytoecdysteroids. For example, in *Ephestia kuehniella* Zeller (Sahaf and Moharramipour,

Unpublished Data), *Plodia interpunctella* Hubner (Lepidoptera: Pyrallidae) (Rharrabe *et al*., 2009) and *Acrolepiopsis assectella* (Zeller) (Lepidoptera: Acrolepiidae) (Arnault and Slama, 1986). In these insects after ingestion of ecdysteroids, larval development disrupts. Also, ingestion of *Serratula tinctoria* L. (Asteraceae) extracts, a plant containing phytoecdysteroids, affects the development and sex ratio of *Lobesia botrana* (Denis and Schiff) (Lepidoptera: Tortricidae). As males appeared more sensitive to extracts, sex ratio was significantly modified on treated diets. On the control diet, the sex ratio was favorable to males (Mondy *et al*., 1997). Dorn and Buhlmann (1982) reported that, application of exogenous ecdysteroids in insect's diets reduces fertility of *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae).

According to Rharrabe *et al*., (2009), larval growth and developmental defects could result from the cytotoxicity of phytoecdysteroids on the midgut of larvae. In *Bombyx mori* L. (Lepidoptera: Bombycidae) larvae (Tanaka and Yukuhiro, 1999) and *P. interpunctella* larvae (Rharrabe *et al*., 2010) that fed on 20-hydroxyecdysone, the morphology of midgut epithelial cells was disrupted.

The effects of ecdysteroidal extract on developmental parameters of *P. xylostella* were similar to those for *P. interpunctella* fed by 20-hydroxyecdysone (Rharrabe *et al*., 2009; Rharrabe *et al*., 2010).

Table 1 Reproductive parameters of *Plutella xylostella* on leaves treated with *Spinacia oleracea* ecdysteroidal extract.

Concentration (%)	Parameters (Mean ± SE)[1]			
	Gross fecundity rate (eggs/female/generation)	Net fecundity rate (eggs/female/generation)	Gross reproductive rate (eggs/female/generation)	Sex ratio (Female/Male)
Control	121.255 ± 0.540[a]	111.493 ± 0.513[a]	118.830 ± 0.530[a]	0.47
12.75	66.611 ± 0.717[b]	57.773 ± 0.622[b]	60.016 ± 0.646[b]	0.51
16.96	58.611 ± 0.461[c]	50.506 ± 0.416[c]	52.750 ± 0.415[c]	0.62
20.84	37.505 ± 0.637[d]	28.457 ± 0.502[d]	30.529 ± 0.518[d]	0.67
$F_{(3, 70)}$	3531.55	22117.06	25394.14	
P-value	< 0.0001	< 0.0001	< 0.0001	

1. The means followed by different letters within a column are significantly different (Tukey's test, P < 0.05).

Table 2 Population growth parameters of *Plutella xylostella* on leaves treated with *Spinacia oleracea* ecdysteroidal extract.

Concentration (%)	Parameters (Mean ± SE)[1]				
	R_0 (female/generation)	r_m (day^{-1})	λ (day^{-1})	DT (day)	T (day)
Control	56.495 ± 4.864[a]	0.311 ± 0.008[a]	1.364 ± 0.011[a]	2.221 ± 0.061[b]	12.981 ± 0.165[c]
12.75	30.622 ± 5.930[b]	0.245 ± 0.013[b]	1.277 ± 0.018[b]	2.817 ± 0.163[a]	14.032 ± 0.209[a]
16.96	32.643 ± 4.572[b]	0.268 ± 0.012[b]	1.307 ± 0.015[b]	2.578 ± 0.117[a]	13.022 ± 0.311[b]
20.84	20.458 ± 4.846[b]	0.255 ± 0.022[b]	1.291 ± 0.028[b]	2.687 ± 0.242[a]	11.902 ± 0.205[d]

1. The means followed by different letters within a column are significantly different (LSD test, P < 0.05).
R_0: Net reproduction rate, r_m: Intrinsic rate of increase, λ: Finite rate of increase, DT: Doubling time, T: Mean generation time.

In summary, this work establishes the potent effects of ecdysteroidal extract on *P. xylostella*. The extract caused significant disturbance to growth and development, confirming that phytoecdysteroids can be a valuable plant defense against insect pests. It would be of interest to check the activity of pure phyoecdysteroids on *P. xylostella* and to perform similar experiments on other species in order to determine the efficacy of the ecdysteroidal extract and phytoecdysteroid.

These results and literature data indicated that ecdysteroidal extract and phytoecdysteroids play a role as defensive substances against insect pests. Possibility of using these compounds for crop protection against insect pests however needs much laboratory and field works.

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اثرات عصاره اکدیستروئیدی گیاه اسفناج *Spinacia oleracea* روی پارامترهای دموگرافیک شب‌پره پشت الماسی *Plutella xylostella* L. (Lepidoptera: Plutellidae)

بی‌بی زهرا صحاف و سعید محرمی‌پور*

دانشگاه تربیت مدرس، دانشکده کشاورزی، گروه حشره‌شناسی کشاورزی، تهران صندوق پستی ۳۳۶-۱۴۱۱۵
* پست الکترونیکی نویسنده مسئول مکاتبه: moharami@modares.ac.ir
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چکیده: امروزه عصاره‌های گیاهی، از جمله فیتواکدیستروئیدها، به‌دلیل توانایی کنترل آفات مورد توجه قرار گرفته‌اند. فیتواکدیستروئیدها، ساختاری مشابه هورمون‌های استروئیدی گیاهان دارند و به روش ضد تغذیه‌ای و یا ایجاد مسمومیت از طریق اختلال در غدد درون‌ریز روی حشرات گیاه‌خوار اثر بازدارندگی دارند. در این پژوهش اثرات عصاره گیاه اسفناج *Spinacia oleracea* L. (Chenopodiaceae) بر پارامترهای دموگرافیک شب‌پره پشت الماسی *Plutella xylostella* L. (Lepidoptera: Plutellidae) بررسی گردید. اسفناج از معدود گیاهان قابل کشت است که حاوی ترکیبات اکدیستروئیدی می‌باشد. لاروهای سن‌سوم شب‌پره پشت الماسی به‌مدت دو روز از غذای تیمار شده با عصاره برگ اسفناج تغذیه کردند. سپس روی برگ‌های سالم تیمار نشده پرورش داده شدند. تخم‌های حاصل از جفتگیری حشرات کامل جهت انجام آزمایشات دموگرافیکی مورد استفاده قرار گرفت. لاروهای تازه خارج شده از تخم در نسل جدید به‌طور جداگانه روی برگ‌های تیمار نشده پرورش یافتند. آزمایش در شرایط دمای ۱ ± ۲۷ درجه سلسیوس، رطوبت نسبی ۵ ± ۶۵ درصد و در شرایط نوری ۱۶ و ۸ (روشنایی و تاریکی) انجام شد. تجزیه و تحلیل داده‌ها نشان داد که باروری، نرخ ذاتی افزایش جمعیت و نرخ خالص تولید مثل با افزایش غلظت عصاره به‌شدت کاهش یافت. اما مدت زمان دو برابر شدن جمعیت با افزایش غلظت افزایش یافت. پژوهش حاضر نشان داد که اثرات گوارشی ترکیبات اکدیستروئیدی اسفناج تنها به‌مدت ۲ روز در مرحله لاروی می‌تواند در نسل بعد حتی با عدم حضور این ترکیبات، پارامترهای دموگرافیک حشره را به‌شدت تحت تأثیر قرار دهد. در نتیجه، این عصاره می‌تواند به‌عنوان یک ترکیب ثانویه مهم برای حفاظت گیاهان در برابر حشرات آفت معرفی گردد.

کلید واژگان: شب‌پره پشت الماسی، *Plutella xylostella*، فیتواکدیستروئید، دموگرافی، پارامترهای بیولوژیک، اسفناج، *Spinacia oleracea*