Research Article

Cold Storage of Predatory Coccinellid *Coccinella septempunctata* L. (Coleoptera; Coccinellidae) to Increase its Shelf-Life for Biological Control Programmes

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Abstract | Ladybird beetles are well acknowledged predators of soft bodied insect pests. Mass production of these beetles for biological control is widely practiced in the world against different insect pests. Many insects can modulate their life processes with respect to low temperatures and this attribute can wisely be used to increase their shelf life for mass rearing. The possibility of short-term storage for adults and larval stages of seven spotted ladybird beetle under low temperature conditions was examined in the present study. The results showed that adults survived well up to five weeks of storage period at two temperature regimes. However, duration of cold storage affected the post storage survival of adult and larvae of *C. septempunctata*. Better survival was recorded in beetles stored at 4°C after their removal from cold conditions. Larval survival declined gradually with increased storage length. Most of the larval instars remain alive under storage conditions up to four weeks, but larval survival in third and fourth instar larvae did not extend after three weeks of storage. Our results provide a guideline to extend shelf-life of C. *septempunctata* for its use as natural enemy in biological control programmes. Such studies may help in planning the storage, transportation and release of these useful natural enemies in the IPM programmes.

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Introduction

Arthropods have progressed through a variety of physiological, morphological and behavioral changes for their development and survival with respect to environmental conditions (Ramløv, 2000; Terao *et al*., 2012). Among environmental conditions day length, temperature, and food quality trigger various responses to insect life histories (Bale and Hayward, 2010; Abarca, 2019). Temperature is the most significant deriving factor in the distribution and mortality of insects in an environment as it effects through physiological processes and further determines the ecological outcomes (Bale, 2002).

Fluctuating temperatures within tolerant ranges generally improve the survival and life history traits of insects (Colinet *et al*., 2015). Previous contributions of many authors have increased the knowledge of insect's cold-hardiness by explaining series of developmental and metabolic processes such as adjustment of cryoprotectants for shorter and longer period under variable temperature (Danks, 2007) and cold acclimation effects on decreased mortality of developmental stages in potato tuber moth *Phthorimaea operculella* (Hemmati *et al*., 2014). Exposure of *Spodoptera exigua* to extreme temperatures; high or low have shown its implication towards thermal tolerance and increased survival of different life stages (Zheng *et al*., 2011). Under low temperature, increase in storage duration of *E. Sophia* pupae may results in decreased percentage emergence and longevity in *E. sophia* (Kidane *et al*., 2015) and storage of *E. mundus* at 5 and 10 ˚C for 4, 8 and 10 days did not effected the parasitism ability on *B. tabaci* (Kazak *et al*., 2020). Studies also found possibility of cold storage of five different Aphidiine parasitoids for short period without any appreciable loss of their life history traits (Colinet and Hance, 2010). For the effective mass production of beneficial insects (predators or parasitoids), it is necessary to develop appropriate methods and approaches for their storage under conditions without any negative effects on fitness and quality parameters of these organisms (Bertanha *et al*., 2021). Therefore, increased shelf life of biocontrol agents (BAs) is the prime and foremost requirement for the timely availability in the fields (Baker *et al*., 2020) for a constant supply (He *et al*., 2019), to meet a pest outbreak (Bielza *et al*., 2020) or to meet fluctuating demand and requirement in the field (Bale *et al*., 2008).

Generally, temperature range from 3 to 6°C is considered suitable for cold storage of the ladybirds (Ruan *et al*., 2012), but ecological or behavioral adaptations may vary for Coccinellids inhabiting different ecological zones. Storage of insects at temperature below their normal threshold temperature has proved successful for short period of time (Gagne´and Coderre, 2001). For instance, larvae of *Coleomegilla maculata* lengi Timberlake has been stored successfully for first two weeks below their thermal threshold of developments and their survival in storage was about 100% for the initial two weeks (Gagne´and Coderre, 2001); irradiated larvae of *C. septempunctata* for 14 days at 8°C (Hayat *et al*., 2020) and eggs of zigzag beetle *Menochilus sexmaculatus* were stored for 3 days at 4°C, 8°C, and 12°C (Tian *et al*., 2021). Significant decrease in the survival of adult beetles *H. variegata* was reported with increasing storage duration at temperatures 1°C and 6°C and an alternating temperature regime of 12°C and 0°C (Sakaki *et al*., 2019).

Development of cold hardiness in most of the insects is very much correlated with some structural and functional adaptations such as production and buildup of biological compounds like simple and complex carbohydrates, organic compounds with multiple functional hydroxyl groups and antifreeze proteins (Bhroozi *et al*., 2012; Izadi *et al*., 2019; Lee, 2010; Vrba *et al*., 2017). Function of these metabolites is related with the maintenance of cellular balance in animals and protection from the drastic unfavorable changes in the temperature (Colinet *et al*., 2016; Mischaud *et al*., 2008). Increase in fat reserves, preservation and use in overwintering insects and their selective consumption have been described by Sinclair and Marshell (2018). Under low temperature insects can produce less antifreeze protein and have higher energy reserves to survive, but exposure for longer periods can deplete more of energy reserves and negatively effects the survival (Renault *et al*., 2002). Therefore, sometimes long term storage deteriorates the quality of insects due to depletion of energy reserves; carbohydrates, polyols and fats etc. are over the period of low temperature storage (Sun *et al*., 2021).

Low temperature effects on cold hardiness have been studied in different species of coccinellid beetles (Watanabe, 2002; Koch *et al*., 2004; Berkvens *et al*., 2010), especially for *C. undecimpunctata* L. (Abdel-Salam and Abdel-Baky, 2000), *Harmonia axyridis* (Abdel-Salam *et al*., 1977) and *C. maculata* (Gagne´and Coderre, 2001). Cold storage of coccinellid developmental stages; egg, larvae and pupae (Katsarou *et al*., 2005, Awad *et al*., 2013; Wang *et al*., 2013; Tian *et al*., 2021; Hayat *et al*., 2020), effect of low temperature on the survival and sensitivity of different life stages (Amarasekare and Sifuentes, 2012), reproduction, longevity (Mahrjan *et al*., 2017), feeding and movement behaviour (Hannigan *et al*., 2022) have also been recognized.

Heterodynamic processes like diapause and aestivation are well studied for *C. septempunctata* (Hodek *et al*., 2012; Suleman, 2015). Adults of *C. septempunctata* fly to hilltop hibernacula and overwinter in grass tussocks or under stones individually or in clusters of several individuals (Ceryngier, 2000). Even provision of suitable laboratory conditions does not change the behavior of facultative diapause in this species where adults tend to gather within cages in the form of inactive clumps (Suleman, 2015). This seven spotted ladybird has extensively been used in different biological control programmes (Yuanxing *et al*., 2020). As with many other coccinellid predators, both adult and larval stages of seven spotted ladybird are voracious feeder of soft bodied insects. Larval stages are usually less mobile and practically can stay at the site of release for longer periods than adults, therefore, could play an important role in IPM programmes. But there are no substantial studies that report the cold storage of larval instars for and its effect on their subsequent survival of efficiency. A recent study has reported cold storage of up to two weeks for *C. septempunctata* larval instars after exposure to different radiation doses (Hayat *et al*., 2020). Application of cold storage to *Semiadalia undecimnotata* larvae and the temporary storage of different larval instars of the *C. maculata*, have been reported previously (Gagné and Coderre, 2001). Moreover, another study has reported that for larvae of *C. undecimpunctata* cold storage at 5°C show a decreased survival with increase in storage duration (Abdel-Salam and Abdel-Baky, 2000).

Improved methods for bulk storage of this useful predatory coccinellid can ease the production and availability of higher numbers for applied biological pest control in field crops, vegetables and fruits orchards. With this objective, in present study the suitability of different growth stages; adult and larvae of seven spotted ladybird beetle for short term cold storage is assessed in order to facilitate its long-distance shipment, sale, or maintenance of sufficient numbers for field releases at peak times through reduced rearing expenditures and increased capacity to meet demands from IPM practitioners and suppliers. Moreover, such studies will also be helpful in the maintenance of stock culture for regular use and long-term storage studies related to biological and ecological research in future.

Materials and Methods

Adults and larvae of seven spotted ladybird *C. septempunctata* were taken from the stock culture maintained in the IPM/ biological control laboratories of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad. Cabbage aphid *Brevicoryne brassicae* Linnaeus (Hemiptera; Aphididae) was used to feed both adult and larvae of the experimental coccinellids. Both adults and larvae of *C. septempunctata* were stored at two selected low temperature regimes i.e., 4°C and 8°C. Water to adults was provided through a cotton wick soaked in water and no water feeding was provided to larvae during the experiment.

Five groups of adult beetles and 2nd, 3rd and 4th instar larvae were subjected to low temperature treatments (4°C and 8°C) for five consecutive weeks. First instar larvae being very weak and vulnerable without any diet were not considered for this experiment. Pre-fed vigorous larvae of subsequent instars were used. At the end of one week, first group of beetles and respective larval instars were taken out of their respective temperature regimes of cold storage. In the same manner, second group was taken out after two weeks of cold storage from respective temperature. Similarly, all five groups were out of the cold conditions at one week's interval till the end of fifth week. Experiment was performed in triplicate with 10 adults and respective larval instar per replicate in transparent plastic containers of size 4.5cm x 3.5cm. These containers were arranged in trays and placed in cold incubators that were set at two temperatures described above. Beetles and larval instars were taken out of the cold storage in their respective week and later on left under controlled laboratory conditions (temperature 25±2°C, humidity 65% and photoperiod of 14hrs light and 10 hrs darkness). Adults and larvae were provided with alive aphids to feed ad libitum and data were recorded for their post-cold storage period survival. Observations were recorded for the length of duration each instar took to complete its development up to adult stage.

Statistical analysis

Data were subjected to statistical analysis following Statistical programme SPSS ver. 16.0. Normality of the data was checked following One-sample Kolmogorov-Smirnov test and non-normal data were log transformed before analysis prior to parametric variance tests. One- or two-way analysis of variance (ANOVA) for two or more than two variables was performed and LSD test was applied for differences between variables.

Results and Discussion

Results showed that *C. septempunctata* adults can be stored for short term duration (up to five weeks) under cold storage without any significant mortality. However, at both temperatures duration of storage affected the afterward survival of adult beetles (Figure 1). Adults showed a comparatively better survival rate at lower temperature (4°C), once have been removed from the cold conditions (Figure 1).

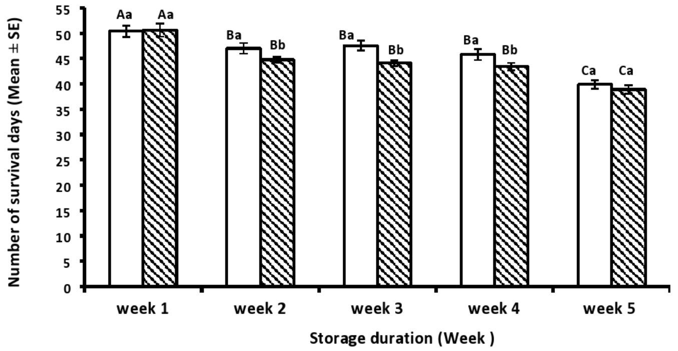

Figure 1: *Week-wise comparison for the survival of adult beetles after removal from cold storage at 4oC (open bars) and 8oC (pattern bars)*

** Capital letters are for differences among weeks at same temperature, whereas small letters indicate difference among beetles at two temperatures for the respective week with standard errors of means.*

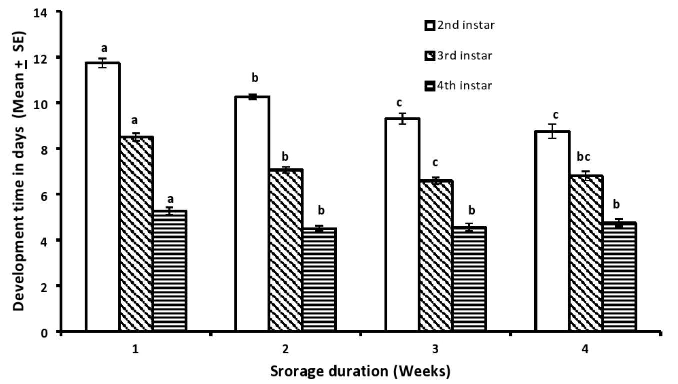

Figure 2: *Week-wise comparison of the development time for the 2nd, 3rd and 4th instar larvae of C. septempunctata after removal from cold storage at 4oC (N = 120) with standard errors of means.*

Storage of various instars of *C. septempunctata* larvae at 4 and 8°C for weekly intervals showed a gradual decline in their survival percentage as the duration of the storage was increased (Figures 2 and 3). Larval survival was hundred percent for first week at both temperatures, it gradually decreased over the period of time and reached to minimum i-e zero on 5th week of cold storage. Third and fourth instar larvae did not survive beyond three weeks at 8°C (Figure 3). Generally, all larval instars survived well but there was gradual decline in their survival (Figure 3).

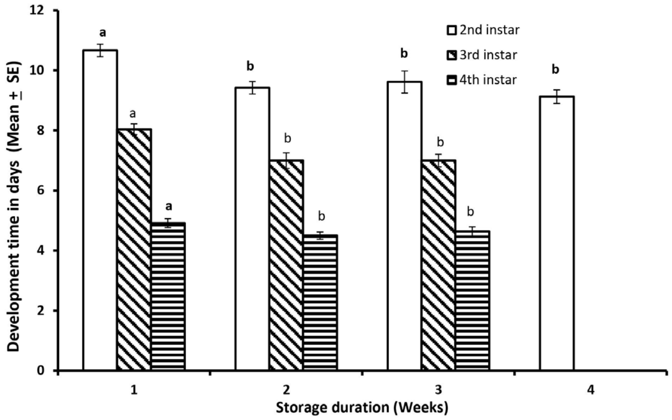

Figure 3: *Week-wise comparison of the development time for the 2nd, 3rd and 4th instar larvae of C. septempunctata after removal from cold storage at 8oC (N = 120) with standard errors of means.*

Developmental time of different larval instars up to pupal stage at both temperatures was significantly affected by the duration of cold storage (Figures 2 and 3). At temperature 4°C and 8°C, all larval instars took longest time to complete their development when stored for one week whereas, developmental time was shortened as the duration of cold storage increased. Significant difference was found for the developmental time at 4°C for different week intervals of storage among 2nd, 3rd and 4th instar larvae (ANOVA 2nd instar, $F_{(3,69)}$= 36.61, P= 0.001; 3rd instar, $F_{(3,63)}$= 27.89, P= 0.001; 4th instar, $F_{(3,66)}$= 5.45, P= 0.002). At 8 °C different larval instars also showed significant difference for weekly storage intervals (ANOVA 2nd instar, $F_{(3,69)}$= 8.07, P= 0.001; 3rd instar, $F_{(2,52)}$ = 9.17, P = 0.001; 4th instar, $F_{(2,52)}$ = 2.48, P= 0.094). However, 3rd and 4th instar larvae did not survive beyond three weeks of cold storage at 8 °C. The larval development time was longest for the second, third and fourth instar larvae taken out after one week of cold storage when compared to second and third week of cold storage at 8°C.

On second instar larvae effects of temperature difference were more pronounced, as they showed significant difference at 4°C and 8°C for first two weeks of storage (ANOVA, week 1, $F_{(1,59)}$ = 13.65, P = 0.001; week 2, $F_{(1,37)}$ = 13.24, P = 0.001) but as the duration of storage was prolonged no significant difference was observed for the third and fourth week of storage at both temperatures for 2nd instar larvae (ANOVA, week 3, $F_{(1,59)}$ = 0.51, P = 0.48; week 4, $F_{(1,15)}$= 0.95, P= 0.35). There was an increase in the subsequent development time for the larvae stored at 4 °C up to two weeks of storage time.

The third instar larvae were less sensitive at both temperatures and there were no significant difference for the development time after removal from storage of one, two and three weeks (ANOVA, week 1, $F_{(1,54)}$= 3.60, P= 0.063; week 2, $F_{(1,30)}$= 0.045, P = 0.833; week 3, $F_{(1,25)}$= 2.46, P= 0.129). Similarly, the fourth instar larvae were again not sensitive and the development times were same for one, two and three weeks at both temperatures with non-significant P values (ANOVA, week 1, $F_{(1,53)}$ = 2.37, P = 0.129; week 2, $F_{(1,37)}$ = 0.001, P = 1.00; week 3, $F_{(1,19)}$ = 0.12, P = 0.731). We can see from results that increase in cold storage duration can cause changes in the development time for larval instars and usually the bigger sizes are less affected.

Ideal cold storage temperature and storage duration provide a sound and adequate supply of entomophagous insects for augmentative releases of insect biological control in field crops (Seyahooei *et al*., 2018). Present study for the cold storage of the predatory coccinellid ladybird *C. septempunctata* may help in developing biological pest control programmes by coordinating with bulk production and the time of field releases. We stored seven spotted ladybird beetles at two different temperatures; 4 and 8°C. Previously cold storage of ladybirds at 3 to 6°C was consider suitable without any loss of their fitness (Ruan *et al*., 2012). Certain biochemical conversions and physiological adaptations at varying temperatures sometimes help the individuals to resist low temperature (Sheikh *et al*., 2017; Zuokun *et al*., 2016). Leopold (1998) reported that storage temperature of 3°C to 15°C is helpful in maintaining a particular stage of stored insects for overcoming the difficulties of developmental changes within a specific population used for field release. This temperature range may keep the quiescent state of stored insects intact and curtail the losses during transportation to the end-users.

After appropriate storage temperature, duration of storage is also very critical. Present study shows that it is possible to stockpile the adult beetles (*C. septempunctata*) at low temperatures (4°C and 8°C) up to 5 weeks when short-term storage is required without any significant mortalities or loss of fitness. The post cold storage survival of the beetles showed a slight decline as the time of storage was increased. Similar kind of observation were reported by Sakaki *et al.* (2019), where more than 90% of the aphidophagous predator ladybird beetle, *Hippodamia variegata* females survived for 35 days at temperatures 6°C; however, the surviving proportion decreased with increasing duration of storage period from above 35 days to 60 days. A study with another coccinellid *H. axyridis* showed that survivals significantly decreased with prolonged storage (Sun *et al*., 2021). From all these studies we can see that usually a short-term storage under cold conditions is considered safe for the survival or further development of coccinellids. However, extending the duration of cold storage could sometimes become harmful for most of the insects including the Coleopterous predacious beetles. For example, in *C. maculata*, when larvae exposed to low temperature for longer duration it killed about 40 % of the total larvae (Gagné and Coderre, 2001). For another predatory coccinellid *H. axyridis* (Pallas), Ruan *et al.* (2012), reported significant decrease in survival of adult ladybird beetle when storage duration was increased. Longer durations of cold storage are not safe and may cause increased levels of certain toxins within insect bodies or direct damage in the metabolic pathways of insects (Sun *et al*., 2021). Negative effects on the survival of *Eretmocerus corni* pupae were recorded when stored at low temperature of 4.5±2 °C and 11.5±2 °C for variable duration from 7 days to 24 days (Lopez and Botto, 2013). Sohail *et al*. (2019) reported complete failure in hatching of *Chrysoperla carnea* eggs under cold storage after 18 days. However, in present studies period of storage was done for five weeks and the survival rate of adult beetles was also satisfactory hence it is to be considered as short-term storage.

Cold storage of larval developmental stages for longer time periods is also not safe because the immature developmental stages are weaker than their fully mature adults due to presence of strong protective mechanisms. Present study shows that developmental time of larval instars could be reduced as a result of cold storage for longer periods. Also, a steady decline in their survival was observed with increased time of storage. Another study reported decreased survival rate when duration of cold storage increased more than two weeks for the coccinellid larvae of *C. maculata* (Gagne´and Coderre, 2001). Such decrease in larval survival may be due to changes in the neurohormones responsible for insect development, particularly exposure to unfavorable temperatures for a longer period (Pullin and Bale, 1988). However, short term cold storage of larval instars could have some positive impacts for biological control programmes. As this study indicates that the development time increases after removal from one week of storage, this may further influence the subsequent feeding potential of larvae. A previous study has reported that the host consumption of *C. septempunctata* larvae was enhanced after subjecting to cold storage conditions (Hayat *et al*., 2020). The same study suggested that such behaviour of larvae shows starvation due to absence of feeding during cold storage and subsequent increased hunger level was evident. To elaborate such behaviour, studies of Watanabe (2002) and Sinclair *et al*. (2018) further explained that the adult or developmental stages of insects show decreased metabolic activities under low temperature to keep reserves of their body fat for sustaining themselves and to survive. This may result in better feeding capacity of larvae stored at low temperature (Chapman, 1998; Mccue, 2010). For coccinellid *C. maculata*, improved feeding of larvae was observed after cold storage at 8°C (Gagné and Coderre, 2001). Similarly, the multicolored Asian ladybird, *H. axyridis*, showed higher predatory efficiency with increased feeding potential on host aphid (Ruan *et al*., 2012). Also, keeping *C. septempunctata* adults under starvation has shown some changes in their predation (Suleman *et al*., 2017). Our study indicates that the larval development was prolonged just after one week of cold storage; application of such larvae in field could enhance their effectiveness for a longer period of time in the management of target pests by consuming more number of target pests.

In present study 2nd, 3rd and 4th larval instars of *C. septempunctata* were selected for cold storage in contrary to 1st instar because of their bigger size and physical strength. Previous study regarding cold storage of *C. undecimpunctata* found that cold storage of 3rd and 4th larval instars was higher and much safer than younger larval instars (Abdel-Salam and Abdel-Baky, 2000). The same study showed that only short term cold storage was safe as survival was decreased at greater rate after period of two weeks and complete mortality was recorded after 30 days of cold storage. Likewise, the present study showed that *C. septempunctata* adult and larvae could be stored for short term without any severe impact on their development or afterward survival. Such flexibility for tolerating low temperature conditions can be helpful in different mass rearing techniques of beneficial coccinellids (Zeng *et al*., 2020). In *C. septempunctata* diapause is a conspicuous feature and beetles pass through facultative diapause even under suitable environmental conditions when feeding regime is changed (Suleman, 2015). As *C. septempunctata* is considered one of the strong predators (Bilashini and Singh, 2009; Xia *et al*., 2018), manipulations can made in cold storage practices for its maximum use as biocontrol agent in IPM programmes.

Low temperature tolerance under cold storage is affected by number of factors (biotic and abiotic) experienced by the organism in the course of low temperature exposure also they affect the ability to withstand low temperature before and after cold exposure (Rathee and Ram, 2018). Present cold storage studies may also help to understand the possible mechanisms and adjustments of *C. septempunctata* individuals for their fitness in the changing surrounding environmental temperature. In many insects resistance to unfavorable cold conditions has been improved by exposure to low temperature through physiological changes (Overgaard *et al*., 2005; Facon *et al*., 2017; Feng *et al*., 2018). Previous researchers have also showed the influence of diapause; a physiological phenomenon, for cold tolerance improvement in Colorado potato beetles even without prior exposure to cold (Boiteau and Coleman, 1996).

Reduced emergence, lifespan and reproduction following a cold storage period may occur sometimes (Lins *et al*., 2013; Sakaki *et al*., 2019). Our results showed that afterward survival of adult ladybird beetles was dependent upon the length of storage period. As the storage duration increased, the survival of the beetles (days) was reduced after removal from cold storage conditions. However, intensity of these negative effects on the biotic parameters of insects under storage is mostly proportional to the period of low temperature (Zeng *et al*., 2020). Therefore, people involved in the bulk production of these beneficial insects may have the choice to keep particular species in cold storage for length of period till it meets the basic requirements.

Conclusions and Recommendations

The outcome of the current study shows that storage of adult and larvae of *C. septempunctata* at low temperature for a short time period could have broader application in different biocontrol programmes. For practical needs and IPM approach, sometimes it is desirable to hold adults or various developmental stages of insects under short-term cold storage. Such flexibility can be helpful in devising bulk rearing techniques and transportation of natural enemies. To synchronize both the target insect pests and natural enemies, it is important to strengthen the production of biological control agents; predators and parasitoids through procedures of cold storage. Therefore, cold storage of useful commercial biocontrol agents in huge number for timely field application is simple as well as valuable method in beneficial insect rearing programs. The current study was discontinued after five weeks period because of shortage of some inputs. However, further studies for longer duration storage of this beneficial beetle at low temperature could increase the insight details and usage of this technique in insect pest management programmes. The effectiveness of this technique could also be tested for some other predatory coccinellid beetle species.

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Novelty Statement

This article provides a guideline to extend shelf-life of C. septempunctata for its use as natural enemy in biological control programmes.

Author's Contribution

Nazia Suleman was involved in designing the experiment, statistical analysis, interpretation of data, obtaining funding, coordinating the research as supervisor of Quran ul Ain and Asifa Khan who completed the experimental work and compilation of data. Asia Riaz was involved in editing and drafting of the manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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