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Effect of gamma irradiation and subsequent cold storage on the development and predatory potential of seven spotted ladybird beetle *Coccinella septempunctata* Linnaeus (Coleoptera; Coccinellidae) larvae

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ABSTRACT

Seven spot ladybird beetle, (*Coccinella septempunctata*) is a widely distributed natural enemy of soft-bodied insect pests especially aphids worldwide. Both the adult and larvae of this coccinellid beetle are voracious feeders and serve as a commercially available biological control agent around the globe. Different techniques are adopted to enhance the mass rearing and storage of this natural enemy by taking advantage of its natural ability to withstand under extremely low temperatures and entering diapause under unfavorable low temperature conditions. The key objective of this study was to develop a cost effective technique for enhancing the storage life and predatory potential of the larvae of *C. septempunctata* through cold storage in conjunction with the use of nuclear techniques, gamma radiations. Results showed that the host eating potential of larvae was enhanced as the cold storage duration was increased. Gamma irradiation further enhanced the feeding potential of larvae that were kept under cold storage. Different irradiation doses also affected the development time of *C. septempuntata* larvae significantly. Without cold storage, the lower radiation doses (10 and 25 GY) prolonged the developmental time as compared to un-irradiated larvae. Furthermore, the higher dose of radiation (50GY) increased the developmental time after removal from cold storage. This study first time paves the way to use radiation in conjunction with cold storage as an effective technique in implementation of different biological control approaches as a part of any IPM programs.

Key word: Gamma irradiations; cold storage, *Coccinella septempunctata* larvae; predatory potential; integrated pest management programme

INTRODUCTION: Nuclear techniques such as gamma radiations have a vast application in different programmes of biological control including continuous supply of sterilized host and improved rearing techniques (Greany and Carpenter, 2000; Cai *et al.*, 2017). Similarly irradiation can be used for sentinel-host eggs and larvae for monitoring survival and distribution of parasitoids (Jordão-paranhos *et al.*, 2003; Hendrichs *et al.*, 2009; Tunçbilek *et al.*, 2009; Zapater *et al.*, 2009; Van Lenteren, 2012). Also, at the production level, such technique may facilitate the management of host rearing, improve quality and expedite transport of product (Fatima *et al.*, 2009; Hamed *et al.*, 2009; Wang *et al.*, 2009). Gamma irradiations can also be used to stop insect's development to enhance host suitability for their use in different mass rearing programs (Celmer-Warda, 2004; Hendrichs *et al.*, 2009; Seth *et al.*, 2009). Development and survival of all insects have a direct connection with temperatures which in turn affect the physical, functional and behavioral adaptations (Ramløy, 2000). Many insects living in moderate regions can survive at low temperature by process of diapause. A temperature between 0 to 10°C may cause some insects to become sluggish and they only become active when the temperature is suitable. Such insects show greater adaptations to flexible temperature regimes for better survival. Many studies have reported this concept of cold-hardiness in insects in general (Bale, 2002; Danks, 2006) and specifically in coccinellid beetles over past years (Watanabe, 2002; Koch *et al.*, 2004; Pervez and Omkar, 2006; Labrie *et al.*, 2008; Berkvens *et al.*, 2010). Using this cold hardiness phenomenon, many coccinellids have been studied for the effect of cold storage such as *Coccinella undecimpunctata* (Abdel-Salam and Abdel-Baky, 2000), *Coleomegilla maculata* (Gagné and Coderre, 2001) and *Harmonia axyridis* (Watanabe, 2002). This natural phenomenon, therefore, can be a helpful tool in developing low temperature stockpiling for improving mass-rearing procedures (Mousapour *et al.*, 2014). It may provide a significant output in terms of providing natural enemies as and when required during pest infestation peaks (Venkatesan *et al.*, 2000).

Use of irradiation in conjunction with cold storage proves to be an effective technique in implementation of different biological control approaches as a part of any IPM programme. A study reported that the pupate of house fly, *Musca domestica* irradiated at dose of 500 Gy and can stored up to 2 months at 6°C for future use for a parasitoid wasp *Spalangia endius* rearing (Zapater *et al.*, 2009). Similarly, when irradiated at 20 GY, parasitic wasps *Cotesia flavipes* were stored safely up to two months without deterioration of their parasitic potential (Fatima *et al.*, 2009). Similarly, bio-control program of sugarcane shoot borer *Chilo infescatellus* proved successful through the use of irradiation combined with cold storage of its egg and larval parasitoids *Trichogramma chilonis* and *C. flavipes* (Fatima *et al.*, 2009). Less mobile life stages such as larvae are of significance in any IPM strategy because they remain on target site for more time period as compared to adults. Therefore, use of predatory larvae is very promising in different biological control approaches because of their immediate attack on pests and more resistance to unfavorable environmental conditions than delicate egg stage. In addition, with their augmentation into fields, larval stage shows their presence for longer time than adult stage and their feeding potential is also satisfactory as that of adults. For the best utilization of these predators in the field and maximum impact of 3rd and 4th larval

instars on prey, we should encourage late 2nd second instar larvae of predatory beetles in the fields as these instars have more feeding capacity due to increased size and ability to handle larger preys.

In spite of higher significance, there is little information available about the effect of cold storage on the survival of larval instars of different ladybird beetles and its effect on their predatory potential. Very few studies report the use of cold storage for non-diapausing larval stage like for *Semiadalia undecimnotata* and only one study reported the short-term storage (up to two weeks) of 2nd and 3rd instar coccinellid, *C. maculate*, without any loss in feeding voracity of larvae after storage (Gagné and Coderre, 2001). The survival of 3rd and 4th larval instars of *C. undecimpunctata* for 7 days after storage at 5°C was reported in a study but the survival rate declined after 15-60 days of storage (Abdel-Salam and Abdel-Baky, 2000). As *C. septempunctata* is considered one of the voracious predators (Afroz, 2001; Jandial and Malik, 2006; Bilashini and Singh, 2009; Xia *et al.*, 2018) and diapause is a prominent feature of this beetle and it may undergo facultative diapause under suitable laboratory conditions (Suleman, 2015). No information is available to date about the combined effect of cold storage and irradiation on the larval instars of this species.

OBJECTIVES: The objective of this study was to devise a cost effective technique for the cold storage and its effect on the subsequent predatory potential of the seven spotted ladybird beetle larvae in conjunction with the use of gamma radiations. Hypothesis of the study was that an optimum length of low temperature treatment for storage purpose would not affect the predation capacity of *C. septempunctata* larvae and their developmental parameters including survival and pupation will remain unaffected. Furthermore, use of gamma irradiation will have some additional effects on survival and feeding capacity of irradiated *C. septempunctata* larvae. Such techniques can be utilized in different biocontrol programs where short term storage is required. So these larvae can be successfully imparted in different IPM programs against sucking complex of insect pests as a component of biological control strategy

MATERIALS AND METHODS: Plant materials: Collection and rearing of *C. septempunctata*: Adult *C. septempunctata* were collected from the wheat crop (in NIAB vicinity and farm area) in the month of March during late winter and early in spring season 2016-2017. They were kept in plastic jars and were fed with brassica aphids. Under controlled laboratory conditions (25±2°C, 16h: 8h L:D and 65±5% R.H.), eggs of *C. septempuctata* were obtained and after hatching, larvae were also given brassica aphids as dietary source. Larvae of second instar were selected for this experiment (as the first instar is generally very weak and vulnerable to mortality under low temperatures). As the larvae approached second instar, they were separated for the experimentation.

Irradiation of larvae at different doses: Irradiation of larvae was carried out by the irradiation source ^{137}CS at Radiation laboratory, and the larvae were then brought back to the IPM laboratory, Plant Protection Division, Nuclear Institute for Agriculture and Biology (NIAB) Faisalabad. Radiation doses of 10 GY (Grey), 25 GY and 50 GY were used to treat the second instar larvae. There were three replicates for each treatment and five larvae per replicate were used. Control treatment was left un-irradiated.

Cold storage of irradiated larvae: In present work, second instar *C. septempunctata* larvae were studied for storage at low temperature of 8°C. The larvae were kept at 8°C for 0, I and II weeks where week 0 depicts no cold treatment and this set of larvae was left under laboratory conditions for feeding and to complete their development. For larvae that were kept under cold storage for one week at 8°C, the term week I was devised. Similarly, week II denotes the larvae that remained under cold conditions (8°C) for two continuous weeks. Larvae were removed from cold storage in their respective week i.e., after week I and week II and were left under laboratory conditions to complete their development by feeding on aphids.

Data collection: For recording the predatory potential of *C. septempunctata* larvae, 100 aphids were provided per larva per replicate on a daily basis until pupation as this number was more than their feeding capacity to make sure that they were not starved (personal observation). Observations were recorded for survival rate, developmental time and feeding potential.

Data analysis: Data were statistically analysed by Statistical Software SPSS (Version 16.0). The data were subjected to normality check through the One-sample Kolmogorov-Smirnov test. Non normal data were transformed to normal data which were then used for all parametric variance tests. One-way and two-way analyses of variance were used. For comparison between variables, LSD test at α 0.05 was applied.

RESULTS: Feeding potential of irradiated larvae after removal from cold storage: Results showed an increase in the feeding potential of *C. septempunctata* larvae with increased cold storage duration. The feeding potential was significantly higher for the larvae that spent maximum length of time (week II) under cold storage conditions followed by week I and week 0. Gamma irradiations further enhanced the feeding potential of larvae that were kept under cold storage.

When larvae were irradiated at 10 GY, the eating capacity of larvae increased significantly with the duration of cold storage. Similarly, larvae that were irradiated at 25 GY, showed increase in feeding potential on aphids as the time period of cold storage increased. The feeding potential of larvae that were irradiated at 50 GY, was again significantly increased with increase of cold storage duration. When different radiation doses were compared to week 0 of storage, there was a significant difference in feeding potential and larvae irradiated at 50 GY consumed the maximum numbers of aphids when no cold storage was done followed by larvae irradiated at 10 and 25 GY. With the other treatment, where larvae were kept under cold storage for one week (week I) the larvae irradiated at 50GY again showed the highest feeding potential. The feeding potential of irradiated larvae was again significantly higher than the un-irradiated larvae that were kept for two weeks (week II) under cold storage (table 1).

Two-way ANOVA was performed to check the interaction between the different radiation doses and different lengths of storage durations for feeding potential of *C. septempunctata* larvae on aphids. The feeding potential of larvae irradiated at different doses and subjected to variable durations of cold storage were significantly different for both the radiation doses and cold storage intervals. Furthermore, the interaction between the radiation doses and storage duration was also significant meaning that the larvae irradiated at different doses with different length of cold storage were having significant

variations in feeding levels (table 2).

	Week 0		Week 1		Week 2		*P*-value (Week-wise)
Dose (GY)	**N**	**Mean±SE**	**N**	**Mean±SE**	**N**	**Mean±SE**	
0	85	88.01±0.19Bc	24	90.11±0.41Bb	24	91.54±0.26Ba	<0.001
10	112	87.54±0.17BCc	18	90.67±0.47Bb	15	94.20±0.33Aa	<0.001
25	111	87.41±0.17Cc	39	90.88±0.31Bb	33	94.27±0.22Aa	<0.001
50	84	90.29±0.19Ac	71	91.70±0.24Ab	40	93.90±0.20Aa	<0.001
P-value (Dose-wise)	-	<0.001	-	=0.007	-	<0.001	-

Table 1: Table 1: Feeding potential of *Coccinella septempunctata* larvae irradiated at different doses (0GY, 10GY, 25GY and 50GY) and storage durations (Week 0, 1 and 2).
Means followed by the same letters are significantly not different at $P < 0.05$ where capital letters are for dose-wise and small letters are for week-wise comparisons.

Source	***df***	***F***	**P**
Doses	3	22.525	<0.001
Weeks	2	216.750	<0.001
Dose × weeks	6	33.093	<0.001

Table 2: Two-way ANOVA of interaction of different radiation doses with storage durations of cold storage for feeding potential of *Coccinella septempunctata* larvae.

Developmental time of irradiated larvae after removal from cold storage: Significant difference was found in the development time of the larvae of *C. septempunctata* when irradiated at different doses at week 0 (without cold storage). The larvae irradiated at 10 GY took the maximum time for development and with the increase in irradiation dosage, from 25 to 50 GY, the time of development was shortened. The larvae irradiated at 50 GY had the same development time as the un-irradiated ones. When, the irradiated larvae were subjected to cold storage of one week duration (week I), their development time after removal from storage condition varied significantly. The larvae irradiated at 25 GY took the maximum time for development followed by larvae irradiated at 50 GY and 10 GY. There was an indication that the development time was extended for irradiated larvae as compared to un-irradiated larvae.

Results also depicted a significant difference in the time taken by irradiated larvae to complete their development after taken out from cold storage of two weeks duration (week II). As the storage time of irradiated larvae increased, the development time was prolonged. Results showed that the larvae that were irradiated at 25 and 50 GY, took the maximum time to complete their development. With the prolonged duration of cold storage up to two weeks (week II), this difference of development time was less evident at lower doses (10 GY). The larvae irradiated at 10 GY showed a significant difference in their developmental duration after being taken out of cold storage conditions of the week 0, I and II. There was no difference in the developmental duration of larvae that were un-irradiated and subjected to different regimes of storage. Un-irradiated larvae were least affected by the duration of storage. With the increase in the storage time, a decrease in the developmental time was recorded. Larvae that were irradiated at 10 GY, took the maximum period to complete their development when no cold storage was done (week 0) followed by week I and II of cold storage. When the larvae irradiated at 25 GY were compared for their development time, there was again significant difference for week 0, I and II of storage duration. Maximum time was taken by the larvae for their complete development when removed from cold storage after one week (week I). With the increase in storage duration the time taken by larvae to complete their development after removal from cold storage reduced.

When the larvae were removed after different lengths of cold storage duration i.e., week 0, week I and week II, there was a significant difference in the developmental time afterwards. Results have shown that the higher dose of radiation, increased the developmental time after removal from cold storage. The larvae irradiated at 50 GY took the longest time to complete their development after removal from cold storage (week I and week II) as compared the larvae that were not kept under cold storage conditions (week 0) (table 3).

	Week 0		Week 1		Week 2		*P*-value (Week-wise)
Dose (GY)	**N**	**Mean±SE**	**N**	**Mean±SE**	**N**	**Mean±SE**	
0	15	5.60±0.13Ca	4	6.00±0.24Ba	4	6.00±0.23Aa	<0.124
10	15	7.47±0.13Aa	3	6.33±0.28Bb	3	5.00±0.26Bc	<0.001
25	15	6.40±0.13Bb	5	7.60±0.22Aa	5	6.60±0.20Ab	<0.001
50	15	5.67±0.12Cb	11	6.45±0.15Ba	6	6.45±0.14Aa	<0.001
P-value (Dose-wise)	-	<0.001	-	<0.001	-	<0.001	-

Table 3: Development time of *Coccinella septempunctata* larvae between different radiation doses (0GY, 10GY, 25GY and 50GY) and storage durations (Week 0, 1 and 2).
Means followed by the same letters are significantly not different at $P < 0.05$ where capital letters are for dose-wise and small letters are for week-wise comparisons.

Interaction between the different radiation doses and different lengths of storage durations for development time of larvae were checked by two-way ANOVA. The development time of larvae irradiated at different doses and subjected to variable durations of cold storage were significantly different for both the doses and cold storage intervals. Furthermore, the interaction between the radiation doses and storage duration was also significant meaning that the larvae irradiated at different doses with different length of cold storage were having significant variations in development times (table 4).

Source	*Df*	*F*	P
Doses	3	13.215	<0.001
Weeks	2	6.445	0.002
Dose × Weeks	6	17.407	<0.001

Table 4: Two-way ANOVA of interaction of different radiation doses with storage durations of cold storage for developmental time of *C. septempuncata* larvae.

DISCUSSION: The present research work indicates the possibility of keeping the larval instars of *C. septempunctata* under cold storage conditions of 8°C for a short duration of around 14 days without affecting its further development and feeding potential. Furthermore, irradiation can enhance the feeding potential and increase the development time of larval instars. This in turn could be a useful technique in mass rearing and field release programmes for biological control through larval instars. Usually temperature range of 8-10°C is an optimal selection of low temperature for storage as reported earlier for eggs two spotted ladybird beetle, *Adalia bipunctata* and the eggs of *C. septempunctata* (Hamalainen and Markkula, 1977), *Trichogramma* species (Jalali and Singh, 1992) and fairyfly, *Gonatocerus ashmeadi* (Hymenoptra; Mymaridae) (Leopold and Chen, 2007). However, a study reported more than 80% survival rate for the coccinellid beetle, *Harmonia axyridis* for up to 150 days at moderately low temperature of 3-6°C (Ruan *et al.*, 2012). So there is great flexibility in coccinellid adults and larvae for tolerating low temperature conditions. After removal from cold storage, larvae showed better feeding potential with consumption of more aphids when compared to normal larvae that were not placed under low temperature conditions. This indicates that when the adult or immature insect stages are subjected to low temperature environment, they tend to reduce their metabolic activity for keeping them alive on the reserves of their body fats and sustain themselves for a substantial length of time under such cold environment. Hereafter, the larval instars that were in cold storage were behaving as if starved for a certain length of time and showed more hunger. This behavior of improved or higher feeding potential of stored larvae has been reported previously (Chapman, 1998). Hence, the feeding potential of *C. septempunctata* larvae significantly increased after cold storage. Gagné and Coderre (2001) reported higher predatory efficacy in larvae of *C. maculata* when stored at the same temperature as in the present study i.e., 8°C. Similarly, Ruan *et al.* (2012) showed that the multicolored Asian ladybug, *H. axyridis*, when stored under cold conditions, had more eating capacity towards aphids *Aphis craccivora* Koch than the individuals that were not stored. Such studies indicate that the higher feeding potential in insects after being subjected to low temperature environmental conditions could be due to the maintenance of their metabolism rate to a certain level while utilizing their energy reserves to the maximum extent (Watanabe, 2002).

The individuals coming out from cold storage are therefore capable of consuming more pray as they were in a condition of starvation and they have to regain their energy loss through enhanced consumption. Furthermore, the starvation in *C. septempunctata* has previously been reported to affect their feeding potential (Suleman *et al.*, 2017). In the present study, the larval development was delayed after returning to normal laboratory conditions. Cold storage affects the life cycle of many insects other than coccinellids. The cold storage of green bug aphid parasitoid, *Lysiphlebus testaceipes* Cresson (Hymenoptra; Braconidae) mummies increased the life cycle 3-4 times. Nevertheless, in current study the development process of stored larvae resumed quickly after taking them out and larvae completed their development up to adult stage. Similar kinds of results were reported for resumption of larval development after removal from cold storage conditions. Such studies only report satisfactory survival rates and development for a short duration of cold storage but as the length of storage is increased, it could become harmful to certain insects. Gagné and Coderre (2001) reported that cold storage for longer period (three weeks) proved fatal for almost 40% of larvae of *C. maculata.* Furthermore, in the same study, the feeding potential of *C. maculata* larvae was also affected beyond two weeks of cold storage due to the loss of mobility after a long storage period. Many studies have reported that longer durations of low temperature conditions can either damage the metabolic pathways of body cells or may increase the levels of toxins within the bodies of insects. Also, low temperature exposure for longer duration may cause specific interruptions in the insect body especially neuro-hormones responsible for insect development, which could be dangerous or even life threatening.

Chen *et al.* (2004) also reported that the biological qualities of parasitized *Bemisia tabaci* pupae on population quality of *Encarsia formosa* were affected negatively with increase in cold storage duration. Similarly, the egg hatchability of green lacewing *Chrysoperla carnea* Stephen was lost completely beyond 18 days of cold storage (Sohail *et al.*, 2019). However, in the present study the cold storage was done for maximum two weeks and it is to be regarded as a short term storage hence the survival rate was satisfactory. Longer periods of cold storage for larvae are not considered safe due to their vulnerable state as compared to adults which are hardier. Also 2nd instar larvae used in the present study for cold storage for being bigger in size and physical stronger than 1st instar. Abdel-Salam and Abdel-Baky (2000) reported that in *C. undecimpunctata* the cold storage of 3rd and 4th larval instars was higher and considered safer than early larval instars. The same study showed sharp decline in survival rate after two weeks and there was no survival beyond 30-60 days of cold storage. The present study showed that short term storage of the larvae of *C. septempunctata* could be done without any loss of their feeding potential or development so the quality of predator remained unaffected. Similar kind of work for many other insects had been reported previously where cold storage technique proved useful without deteriorating the fitness of stored insects. For example, the flight ability of reared codling moth *Cydia pomonella* Linnaeus remained unaffected after removal from cold storage (Matveev *et al.*, 2017). Moreover, a sturdy reported that pupae of a parasitoid wasp *Trichogramma nerudai* (Hymenoptera; Trichogrammatidae) could be safely put in cold storage for above than 50 days (Tezze and Botto, 2004). Similarly, a technique of cold storage of non-diapausing eggs of black fly *Simulium ornaturm* Meigen was developed at 1°C. Another study reported safe storage of a predatory bug insidious flower bug *Orius insidiosus* for more than 10 days at 8°C (Bueno *et al.*, 2014).

In present study without cold storage, the lower doses of 10 and 25 GY prolonged the developmental time as compared to un-irradiated larvae and higher doses of irradiations in conjunction

with cold storage again significantly prolonged the developmental time of larvae when returned to the laboratory conditions. Salem *et al.* (2014) also reported that Gamma irradiations significantly increased the duration of developmental stages (larvae and pupae) in cutworm, *Agrotis ipsilon* (Hufnagel). In another study, where endoparasitic wasps *Glyptapanteles liparidis* were evaluated with irradiated and non-irradiated gypsy moth *Lymantria dispar* larvae for oviposition, it was found that non-irradiated larvae had a shorter time to reach the adult stage as compared to irradiated larvae (Novotny *et al.*, 2003). Both for higher doses with cold storage and lower doses without cold storage extended the larval duration of *C. septempunctata*. In another study when the parasitoid wasp *Habrobracon hebetor* was irradiated at the dose of 10 GY, it resulted in prolonged longevity (Genchev *et al.*, 2008). In the same study, when another parasitoid *Venturia canescens* was irradiated at lower doses of 4GY and 3 GY, it resulted in increased emergence from the host larvae, while gamma irradiations at the dose of 1 GY and 2 GY significantly stimulated the rate of parasitism (Genchev *et al.*, 2008). The current study also indicated higher rates of predation in the form of increased feeding potential of larvae as a result of irradiations at lower doses.

CONCLUSION: The outcome of the current study shows that storage of 2nd instar *C. septempunctata* at low temperature of 8°C for a short duration of about 14 days is completely safe and could have broader application in different biocontrol programs. Such flexibility in storage duration can also assist in different mass rearing techniques and commercial uses. The combination of gamma radiation with low temperature cold storage could be a useful tool in developing different biological pest management programs against sucking insect pests. Incidence of periodic occurrence of both the target insect pests with their predatory ladybird beetles in synchrony is an important aspect that could be further strengthened by cold storage techniques. Therefore, short or long term bulk cold storage of useful commercial biocontrol agents and then reactivating them at appropriate time of pest infestation is a simple but an advantageous method in mass rearing programs. Increased feeding capacity of stored larvae is another edge and hence such larvae may prove more beneficial as compared to unstored larvae. Both cold storage and improved feeding of the *C. septempuctata* larvae can be utilized for implementation of IPM for many sucking insect pests of various crops, fruits and vegetables. Due to some constraints this study could not be continued beyond two weeks but for future directions, higher doses and longer duration periods could further elaborate the understanding and better application of such useful techniques in future IPM programmes on a wider scale. Also, some other predatory coccinellid beetle species can be tested with similar doses and cold storage treatments to see how effective this technique is on other species as well.

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