Mortality Responses of *Spodoptera litura* Following Feeding on *BT*- Sprayed Plants

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Abstract: *Bacillus thuringiensis* delta-endotoxins are safe biological insecticidal proteins whose usefulness has long been recognized. The first commercialized *Bt* insecticidal formulations were composed of spore-crystal preparations derived from wild-type strains. These products generally have a limited insecticidal host range and several genetically modified strains have, therefore, been constructed in this study using conjugation procedure. However, addition of a new plasmids to Bt strains already harboring other genes often resulted in broader - spectrum. *Bacillus thuringiensis* serovar *Kurstaki*, *Bacillus subtilis* and four of their transconjugants were used in this study as a biocontral agents against lepidopterous cotton pest. Bacterial transconjugants were evaluated for their hybrid vigor in relation to the mid parents and better parent. This evaluation was related to survival and mortality percentages induced in *Spodoptera littoralis* larvae. Two groups of bioinsecticides; crystals, crystals + endospores were used to be evaluated in this study. The results appeared that bioinsecticides containing crystals + endospores was more effective than crystals for increasing mortality percentage and reducing survival percentage. This effective was including reduction in the mean number of *Spodoptera littoralis* larvae feeding on leaves sprayed with crystals + endospores. Increasing mortality percentage of crystals + endospores was due to higher toxicological effects than that of crystals. This recommended bioinsecticide biologists to use crystals + endospores in all bioinsecticides formulations. Higher positive efficiency was appeared at 168 h of treatments. Recombinant *Bacillus thuringiensis* was more effective as biocontrol agents against lepidopteran pests at the early instars, because susceptibility was decreased with larval development. This indicated that the first instars were more susceptible to Bt sprayed plants than the later instar stages. The combined effects of crystals + endospores produced higher mortality. This factor was important to be considered in designing resistance management strategies.

Keywords: Crystals, Endospores, Hybrid vigor, Mortality, Recombinant bioinsecticides, survival.

INTRODUCTION

Each year billions of dollars are spent worldwide on insect control in agriculture [1]. Despite this expenditure, up to 40% of a crop can be lost to insect damage, particularly in developing countries [2]. Some of the most damaging insect species belong to the Lepidoptera, the second largest insect order comprised of moths and butterflies. The larval stage of *Spodoptera littoralis* cause major damage to an array of economically valuable crops including cotton, tomato, corn, sorghum and Lucerne [3]. Until recently, broad spectrum chemical insecticides have been the primary control agent for agricultural pests, with about 40% targeted to the control of lepidopteran insects [4]. Over the years the widespread use of pesticides has led to pesticide resistant insects, a reduction in beneficial insect populations and harmful effects to humans and the environment [5]. These problems have led researchers to develop different insect control strategies using both synthetic and natural molecules that are more environmentally friendly.

Bacillus thuringiensis is a naturally occurring gram - positive bacterium commonly present in soil. Various strains of *B. thuringiensis* (*Bt*) are capable of producing crystal (Cry) proteins (delta - endotoxins) or inclusion bodies that have selective insecticidal effects against different groups of insects [6]. Microbial preparations containing *Bt* cry proteins (as well as cell bodies and spores) have been used as foliar sprays in agricultural and forest settings for several decades. Partly because of their selectivity and short half - life, *Bt*-based microbial insecticides are generally considered to have fewer adverse impacts on the environment than synthetic chemicals [5].

Environmental concerns about the extensive use of chemical pesticides, together with the rising cost of discovering useful new molecules, has stimulated interest in the development of environmentally safe and cheap biopesticides such as microbial insecticides. One example of the successful development of such alternative products is *Bucillus thuringiensis (Bt)* which today accounts for more than 90% of the biopesticides used worldwide. The insecticidal properties of this entomopathogenie bacterium are mainly due to the production, during sporulation, of larvicidal proteins that accumulate (up to 25% of the dry weight) as parasporal crystalline inclusions (also called crystals) within the cell [7]. At the end of the sporulation the cells lyse, spores and crystals are liberated. The inclusions produced by *Bt* subspecies are generally composed of several proteins (designated as delta - endotoxins or Cry proteins) each having a narrow activity spectrum.

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There is thus a large family of related delta – endotoxins classified as CryI, II, III, IV, V, etc., depending on molecular relatedness and activity against insect larvae [8]. More than 50 *cry* genes were belonging to more than 20 different classes or subclasses, have now been cloned from different *Bt* strains. However, despite the demonstrated efficacy of such genetically altered *Bt* products, the efficiency and economic production of *Bt* products could still greatly benefit from the construction of engineered *Bt* strains with a broader activity spectrum and producing larger amounts of each of the crystal delta – endotoxins in the strain. Lepidoptera of the Noctuidae family, such as *Spodoptera littoralis,* are important agricultural pests which are poorly susceptible to most insecticides. The *Bt* recombinant strains were constructed in this study *via* conjugation that allows plasmid DNA transfer between strains that is necessary at the first stages of the construction of the recombinant strains.

Bacillus thuringiensis (Berliner) proteins are becoming ubiquitous, highly bioactive substances in the agro-ecosystems worldwide. This is due to an increase in the use of *B. thuringiensis* - based insecticides and the large scale release of various, transgenic crop plants expressing *B. thuringiensis* proteins conferring plant resistance to certain target insect pests. In the currently commercially available transgenic crop plants, *B. thuringiensis* proteins are present throughout most of the plant during most of the growing period. Further, *B. thuringiensis* protein is expressed in relatively high concentrations and, in contrast to *B. thuringiensis* insecticides, in a truncated, activated form. The current and future trends in plant molecular biology is to increase *B. thuringiensis* expression levels in plants with the most dramatic example being the expression of Cry1Ac in tobacco chloroplasts [9]. Consequently, most, if not all, herbivores colonizing transgenic *B. thuringiensis* plants in the field are not lethally affected by *B. thuringiensis* proteins. However, they will ingest plant tissue containing *B. thuringiensis* protein which they may pass on to their natural enemies in a more or less processed form. The ubiquitous and temporally extended availability of *B. thuringiensis* proteins in the field in addition to its modified form of release, makes it necessary to verify and monitor the compatibility of this new pest management strategy with natural enemies. The long-term, agro-ecological safety of the combined use of transgenic crop plants and *B. thuringiensis* insecticides cannot simply be deduced from the past record of safe *B. thuringiensis* insecticide use when *B. thuringiensis* compounds were available in the field only during short periods. The activity of *B. thuringiensis* insecticides declines rapidly in the field within one week [10]. This study was undertaken to evaluate transconjugant efficiency of recombinant *Bt* isolates with potential impact to control cotton leaf worm, *Spodoptera littoralis*, *via* increasing mortality of this pest and reducing leaf damage.

MATERIALS AND METHODS

Microbial Strains

Bacillus thuringiensis serovar *Kurstaki* (NRRL HD-1) and *Bacillus subtilis* (NRRL NRS-744) were obtained from Dr. L.K. Nakamura, U.S. Department of Agriculture, Agricultural Research Service, U.S. Department of Agriculture, Peoria, I Ilinois. Strains were grown on T_3 medium (L^{-1} 3 g tryptose, 1.5 g yeast extract, 0.05 M sodium phosphate, pH 6.8 and 0.005 g $MnCl_2$, according to Ashfaq *et al.* [11], until sporulation was complete. All strains were grown on L agar (per liter 10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar, according to Ashfaq *et al.* [11] at 30^{o}C until they sporulated. Strains were maintained on LB Slape medium consists of; 1% tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.5.

Mass Culturing of *S. litura*

Field collected egg masses of *Spodoptera littoralis* were used to initiate the mass culturing under laboratory conditions. The egg masses were kept in the egg cage. After emergence, first instar larvae were weighted and transferred to *Bt* and non *Bt* leaves in the bottles. The leaves were weighted, changed daily and the faecal pellets removed from the container every 24h. Larvae grown up were weighted daily.

Host Plants

Fresh leaves of *Ricinus communis* were collected daily, squares and middle leaves were used in bioassay experiments. Leaves were cleaned and three grams were weighted and placed in each container daily.

Bacillus thuringiensis Formulations Used in the Experiment

Bacillus thuringiensis containing viable spores were used in liquid formulations at concentrations of 10^8 CFU/ml. Bio – insecticide was applied on 250 ml bottles, as well as, mixed with three grams of leaves as diet for larvae.

Antibiotic Susceptibility Assays

Antibiotic susceptibility was measured by plate diffusion method, according to Collins and Lyne [12] with cultures grown to logarithmic growth phase in nutrient broth of LB medium. Different antibiotics were used with the concentration of 400 µg/ml, according to Roth and Sonti [13].

rfa Mutation

Strains having the deep rough (*rfa)* character should be tested for crystal violet sensitivity [14]. The presence of the *rfa* mutation which permits large molecules such as crystal violet to enter and kill the bacteria was tested. Wild-type strains or strains containing the *gal* deletion are not inhibited because the crystal violet cannot penetrate the cell.

Conjugation

Conjugation was carried out according to Lessl *et al*. [15], by inoculating 10 µl samples of the donor cultures onto the surface of selective medium, previously seeded with 100 µl of the recipient culture. Single colony of transconjugants was picked up and transferred to LB slant agar medium.

Separation of Crystals

Crystals and endospores were collected and purified according to Karamanlidou *et al*. [16]. To purify crystals from spores and cellular debris, samples were sonicated and centrifuged on discontinuous sucrose density gradients (67 to 72 to 79% [wt/vol] sucrose) at 15000 xg for 2 h. Crystal bands and spore pellets were purified by three centrifugations and washed with distilled water. Final pellets were resuspended in small volumes of distilled water and stored at -5°C.

Bioassay of Toxicity

Spodoptera littoralis second instar larvae (mean body weight = 10 mg) was used for toxicity bioassays according to Klanfon and DeBarjac [17] with some modifications. Bacterial cell component of *B. thuringiensis* containing approximately 10^9 crystals and/or spores per milliliter was used with the dilution of 1:1. Larvae of *Spodoptera littoralis* were exposed to *Bt* bioinsecticide *via* dispense 200 µl of the suspension on 2-3 gram of diet surface of *Ricinus communis* [18], and the surface was air - dried. Mortality was recorded daily after 24 h for 6 - 7 days. Surviving larvae from each replicate were pooled and numbered daily [19].

Measuring Transconjugant Efficiency (TE)

Transconjugant efficiency was expressed as hybrid vigor, which calculated according to Winfridus Bakker [20] using the following formula;

TE (Mid parents) = Average P_{F1} - Average P_P/ Mid parents, measured in units of the trait

TE (Better parent) = Average P_{F1} - Average Better parent / Better Parent, measured in units of the trait

P_{F1} = average performance of crossbreds

P_P = average performance of parents lines = $P_1 + P_2 / 2$.

RESULTS AND DISCUSSION

Genetic Markers in Conjugation

Both *Bacillus thuringiensis* and *Bacillus subtilis* as shown in Table **1** were genetically marked using antibiotic (hiconcil) and drug resistance (Crystal violet). *B. thuringiensis* was found to be more resistant to Hiconcil and *B. subtilis* was found to be resistant to crystal violet. The results obtained herein indicated that antibiotic resistance was due to bacterial extrachromosomal elements that carry genes conferring resistance to one or more antibiotics. These elements named plasmids which transfer conjugatively between bacterial species and are significantly involved in the emergence and dissemination of multiple drug resistance.

Table 1: Genetically Marking Bacteria Against Hiconcil and Crystal Violet Expressed as the Presence or Absence of Inhibition Zone

Marking agents	Bacterial strains	
	B. thuringiensis	***B. suBtilis***
Hiconcil	+	-
Crystal violet	-	+

(+), (-) resistance and sensitivity, respectively.

The results indicated that the genotype of both strains were as follows; *Bacillus subtilis* (*Hico*$^-$ *rfa*$^+$) and *Bacillus thuringiensis* serovar *Kurstaki* (*Hico*$^+$ *rfa*$^-$). These results agreed with Campbell [21], who reported that genes located on a circular strand of DNA called an R-plasmid may contain several antibiotic-resistant genes. Both strains were conjugated and four different transconjugants were isolated to be evaluated for hybrid vigor of toxicity against cotton leafworm.

Hybrid Vigor in Reducing Survivors of *Spodoptera littoralis* Larvae

The results diagrammatic in Figure **1** appeared hybrid vigor in reducing the mean number of *Spodoptera littoralis* larvae after feeding on leaves of *Ricinus communis* (gram/day) sprayed with bioinsecticides of *Bt* transconjugants. It is of interest to note that toxicological effects appeared herein was due to cry + endospores than crystals alone. This indicated that crystals + endospores was more effective than crystals for inducing mortality. Higher hybrid vigor was appeared at 168 h of treatment. During the process of spore formation, *Bt* also produces unique crystalline bodies. When eaten, the spores and crystals of *Bt* act as poisons in the target insects. *Bt* crystals dissolve in the intestine of susceptible insect larvae. They paralyze the cells in the gut, interfering with normal digestion and triggering the insect to stop feeding on host plants. *Bt* spores can then invade other insect tissue, multiplying in the insect's blood, until the insect dies. Death can occur within a few hours to a few weeks of *Bt* application, depending on the insect species and the amount of *Bt* ingested. *Bt* products contain the highly specialized protein crystals and dormant spores of bacteria. These are only activated when they are eaten by a susceptible species of insect. Unlike broad spectrum insecticides, *Bt* is highly specific - that is, it affects only certain species of insects and has no effect on others. The results obtained herein indicated that enteric recombinant bacteria of *B. thuringiensis* have important roles in induced killing of Lepidoptera across a range of taxonomy, feeding breadth, and relative susceptibility to *B. thuringiensis*. These results agreed with Obonyo *et al.* [22], who found that transient feeding of *Chilo partellus* on *Bt* maize at the third and fourth instars significantly delayed their development at the instar which exposure took place.

Schoenmaker *et al*. [23] suggested that the ingestion by lepidopteran larvae of sublethal doses of *Bt* toxin prolonged development time by temporarily inhibiting feeding. Continuous exposure to *Bt* toxin prolonged development of *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) while exposure to toxin for shortened durations had no significant effects on larval development time [24]. It therefore seems that larvae can recover from the effects of the *Bt* toxin, following transient exposure. Other lepidopteran larvae that ingest sublethal doses of *Bt* also resume normal development after a few days [25].

Dutton *et al*. [24] reported that when 3rd instars of *S. littoralis* larvae were exposed to *Bt* sprayed plants (such that the effect of the toxin does not persist for long, since the *Bt* spray is quickly degraded, [26]) there were no significant effects on overall larval development as compared to the significant effects noted when plants were reared for four days on *Bt* maize, such that the effect of the toxin was more persistent. Huang *et al*. [27] observed larval development inhibition of *O. nubilalis*, *D. grandiosella* and *Diatraea saccharalis* F. (Lepidoptera: Pyralidae) fed on a diet prepared from *Cry1Ab* protein extracted from *Bt* corn leaves. Similarly, transgenic maize containing *Cry1Ab* delayed larval development of *H. zeae* [28] and *Danaus plexippus* L. (Lepidoptera: Danainae) [29].

The results also agreed with Haggag and Abou Yousef [30], who found that all *Bt* strains, as well as, reference strain had the toxicity to insect larvae, where the percentages of mortality were in the range of 47.5 - 100.0 % and 25.0 -100.0 % against first and second instars larvae, respectively. The percentages of larvae mortality caused by solubilized crystal toxins against first instar larvae of *S. littoralis* were in the range of 2.5 - 98.0%. The best percentage of mortality was obtained with solubilized crystal toxins at concentration of 200 ppm of toxins, where the percentages of mortality were in the range of 55.0 - 98 %.

Hybrid Vigor Related to Mortality of *Spodoptera littoralis* Larvae

The results diagrammatic in Figure **2** appeared that hybrid vigor of recombinant bioinsecticides increased mortality percentage as shown by transconjugant A which appeared higher values of mortality resulted by cry + endosopores than crystals in relation to the better parent. However, transconjugant A appeared higher values in mortality percentage at the treatments of crystals than that treated with crystals + endospores in relation to mid parents at 48 h to 144h. The same trend was also achieved by transconjugant B in relation to midparents from 96h to 144h. However, transconjugant B appeared higher values of hybrid vigor in mortality values in relation to better parent when the larvae was treated with crystals + endospores than crystals at the times from 24h to 96h. Moreover, transconjugant C appeared higher hybrid vigor in mortality percentage in relation to midparents when the larvae was treated with crystals + endospores than that treated with crystals from 24h to 144h. The same trend in relation to midparents, as well as, to better parent, was also achieved by transconjugant D from 24h to 96h. However, transconjugant D appeared higher hybrid

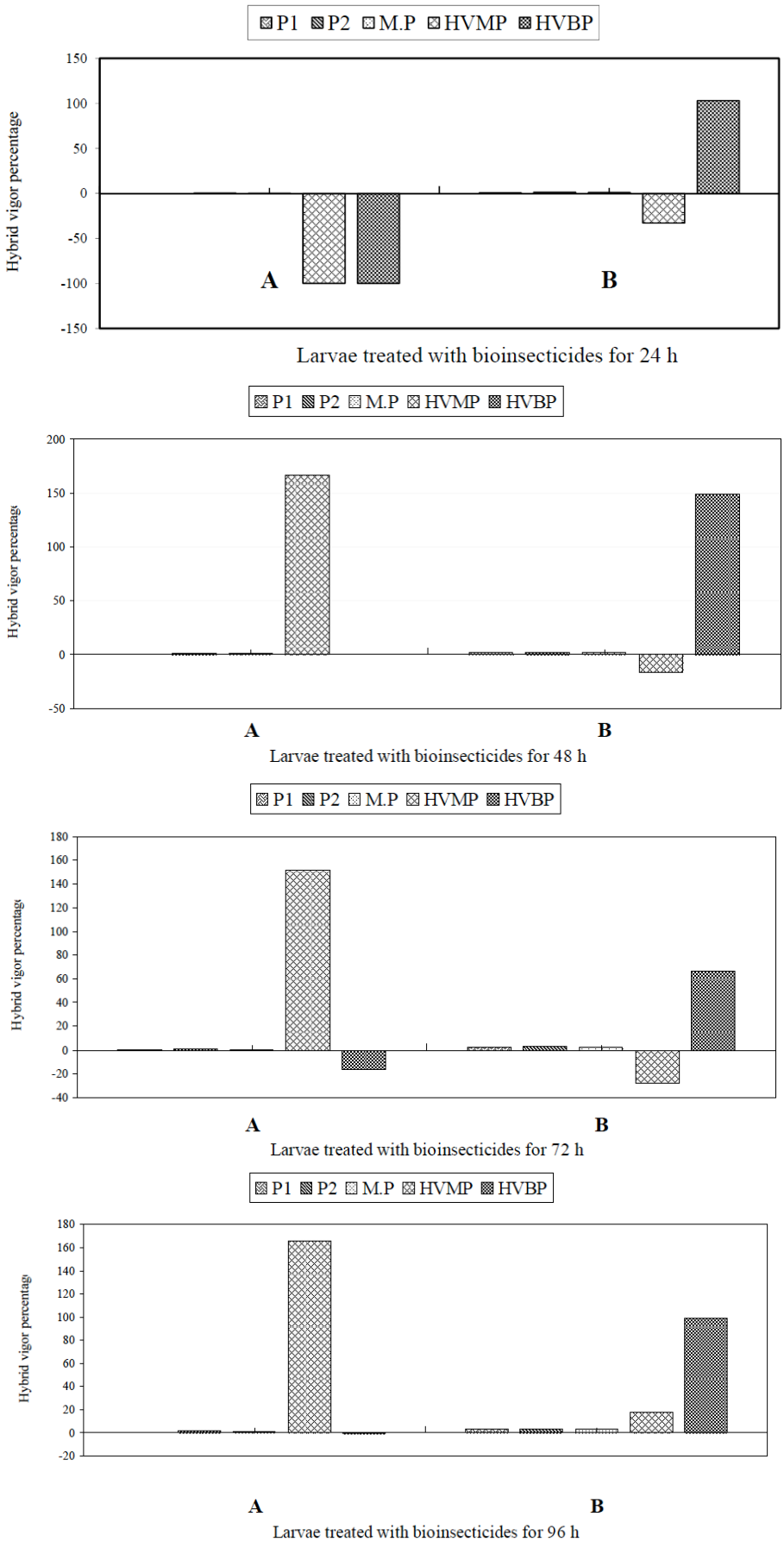


Figure 1: Transconjugant-A.

Figure 1: Continued for transconjugant-A.

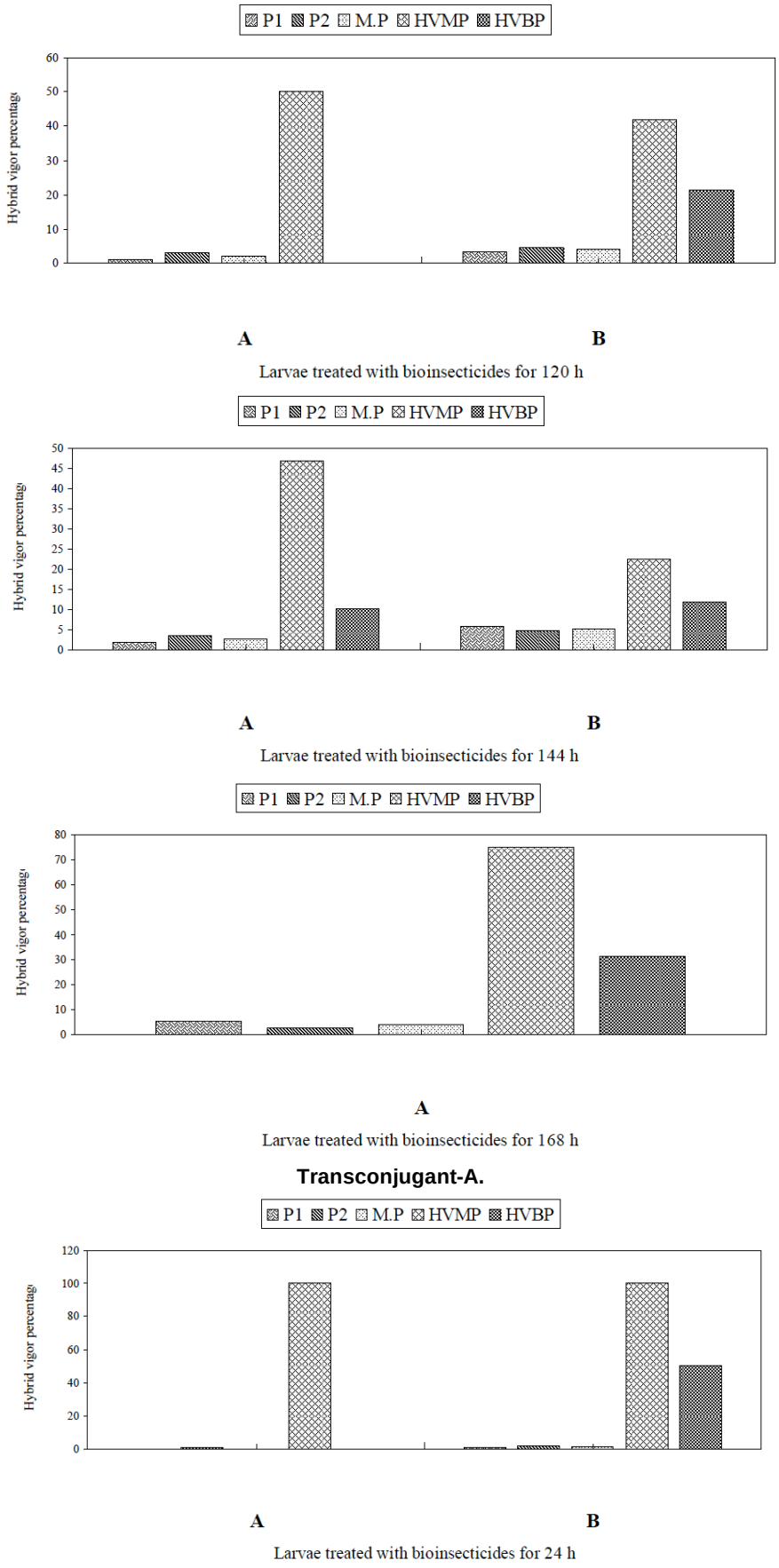


Figure 1: Transconjugant-B.

Figure 1: Continued for transconjugant-B.

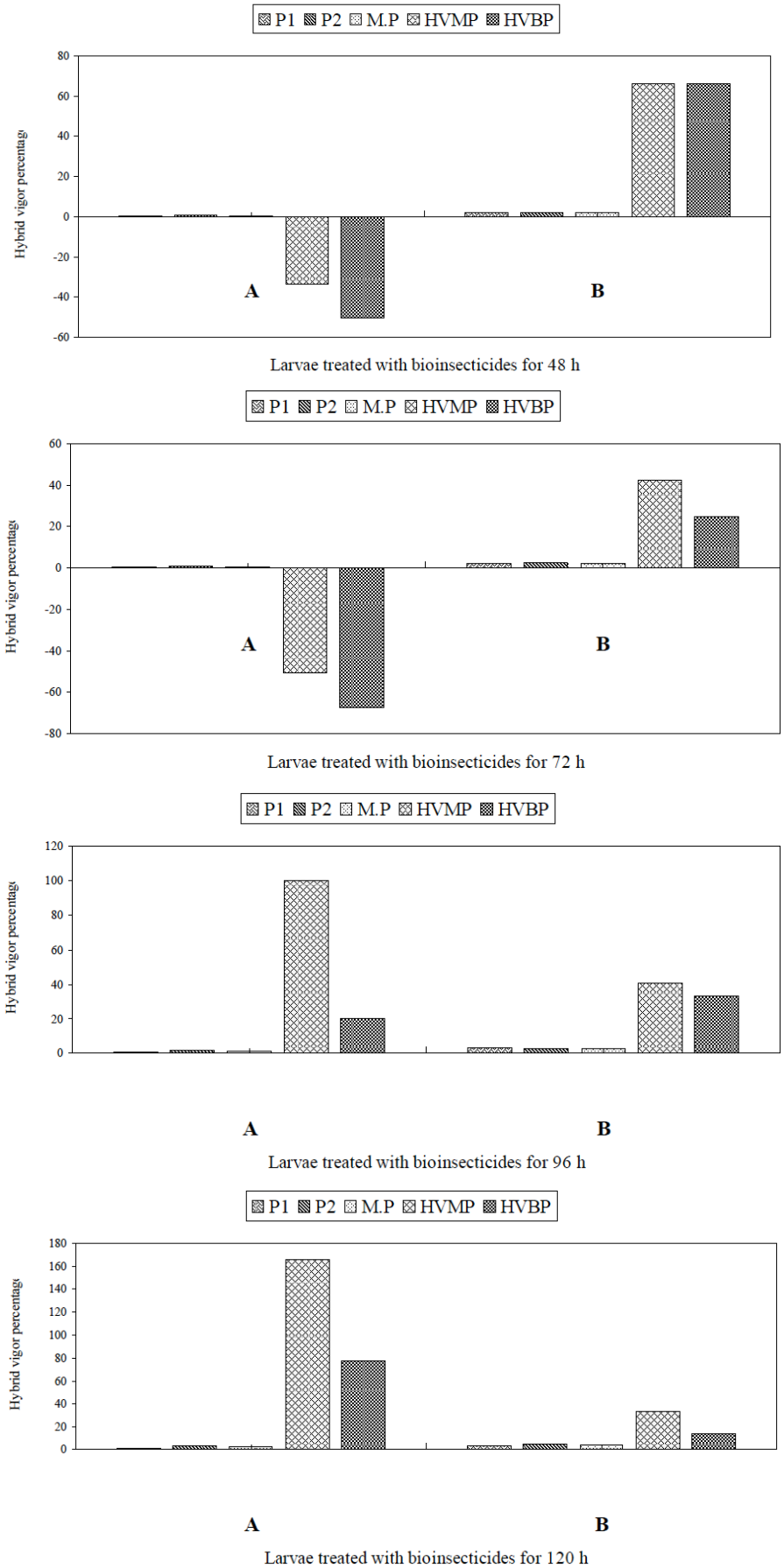


Figure 1: Continued for transconjugant-B.

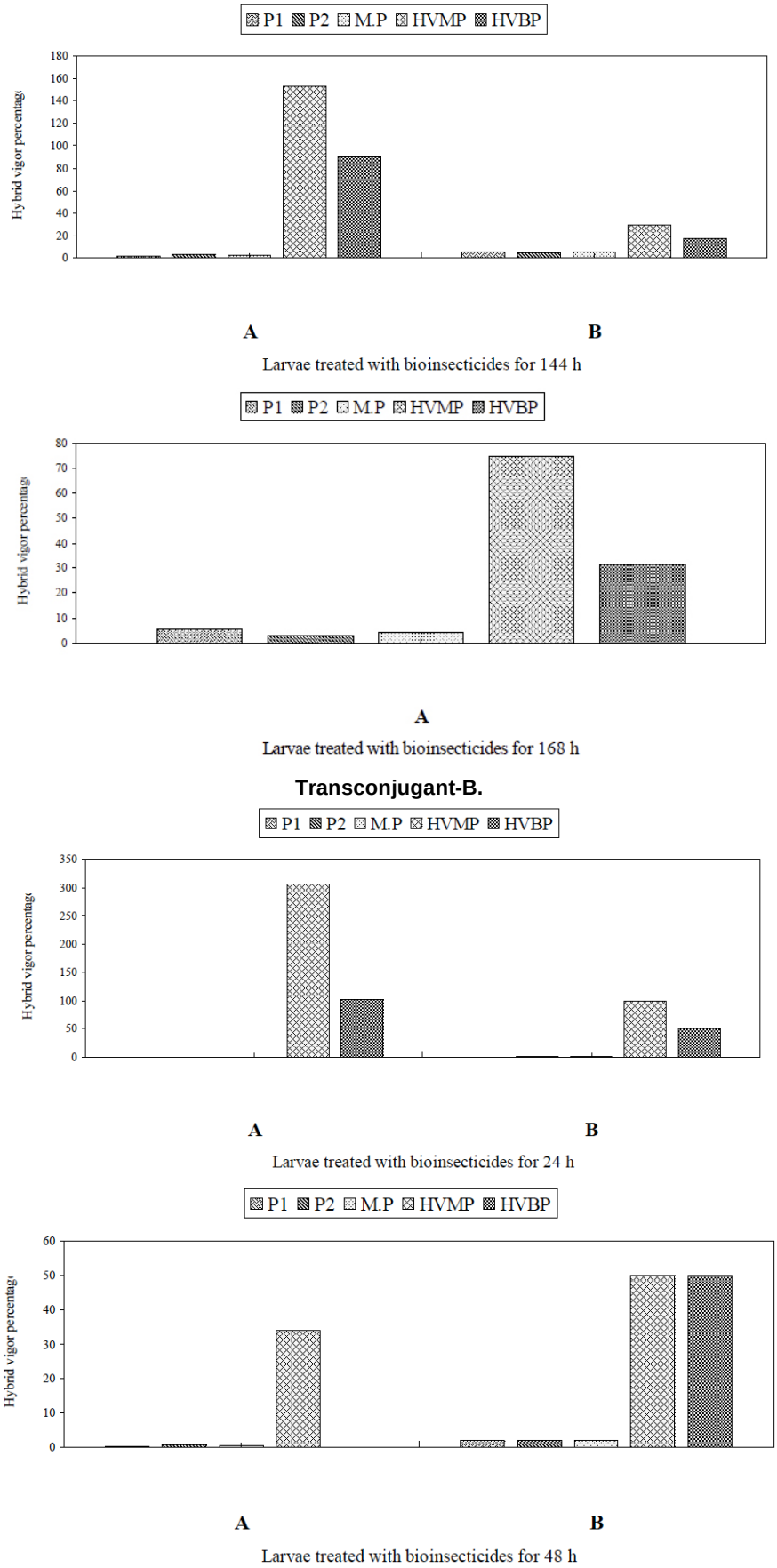


Figure 1: Transconjugant-C.

Figure 1: Continued for transconjugant-C.

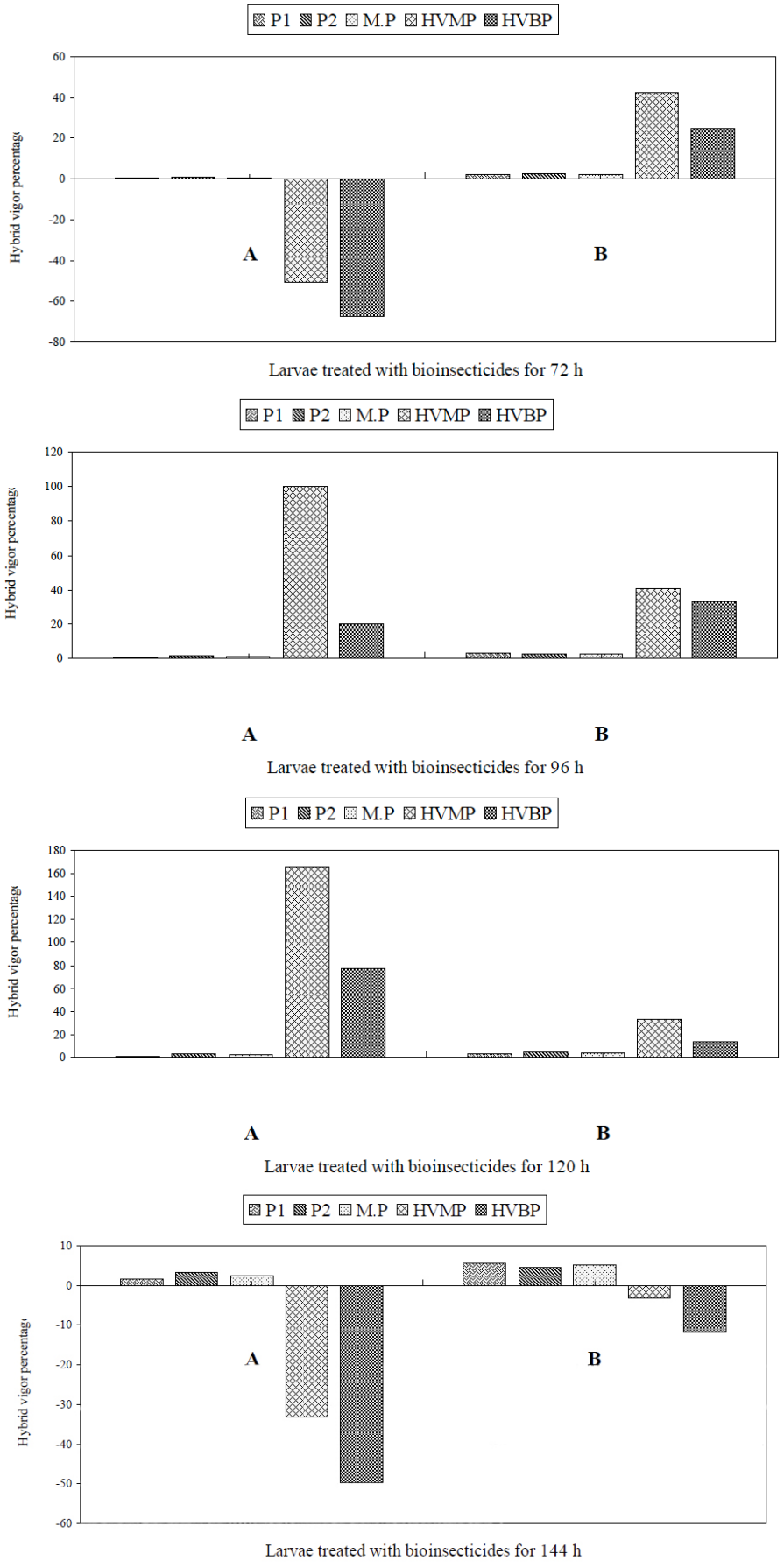


Figure 1: Continued for transconjugant-C.

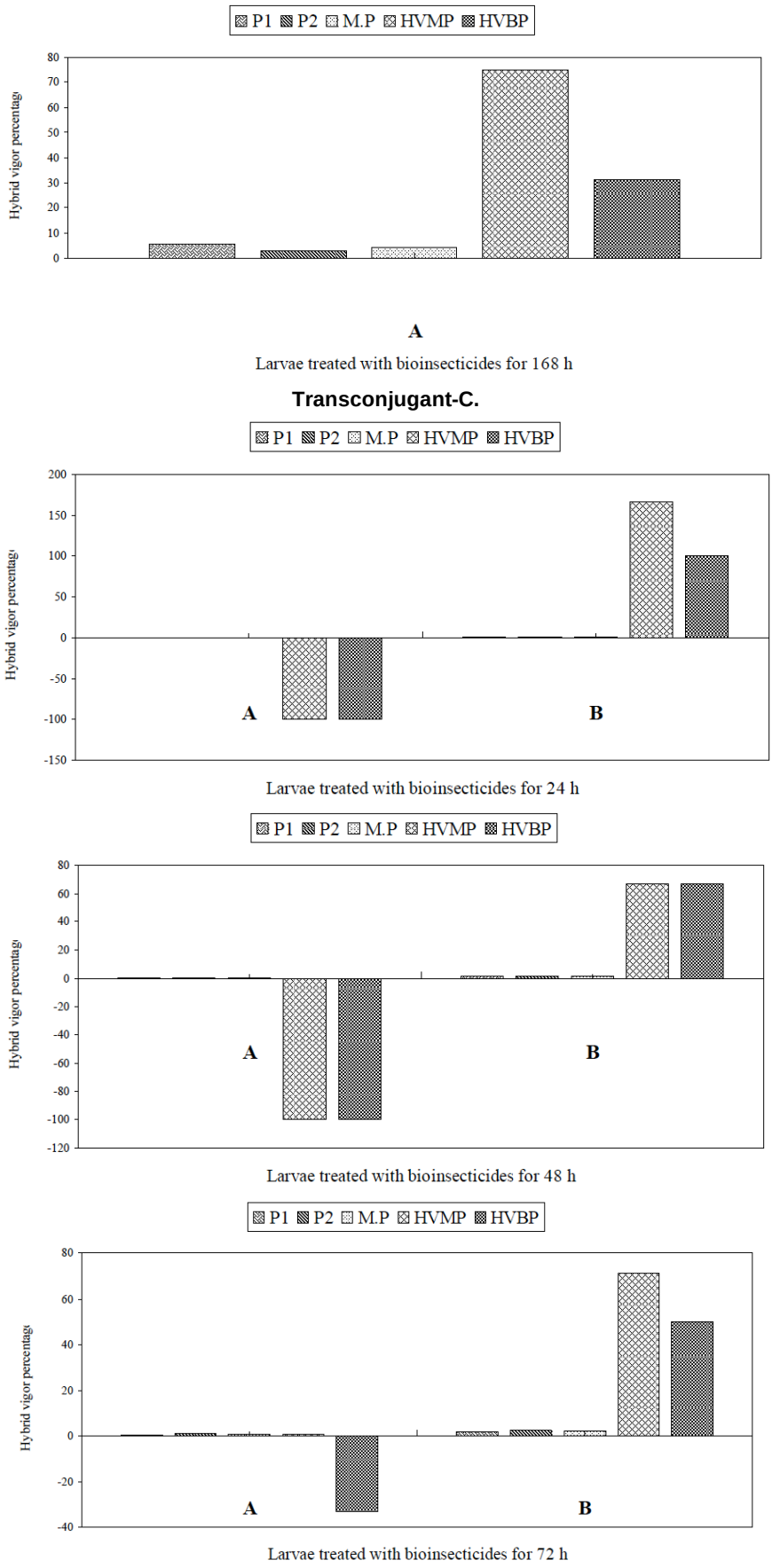
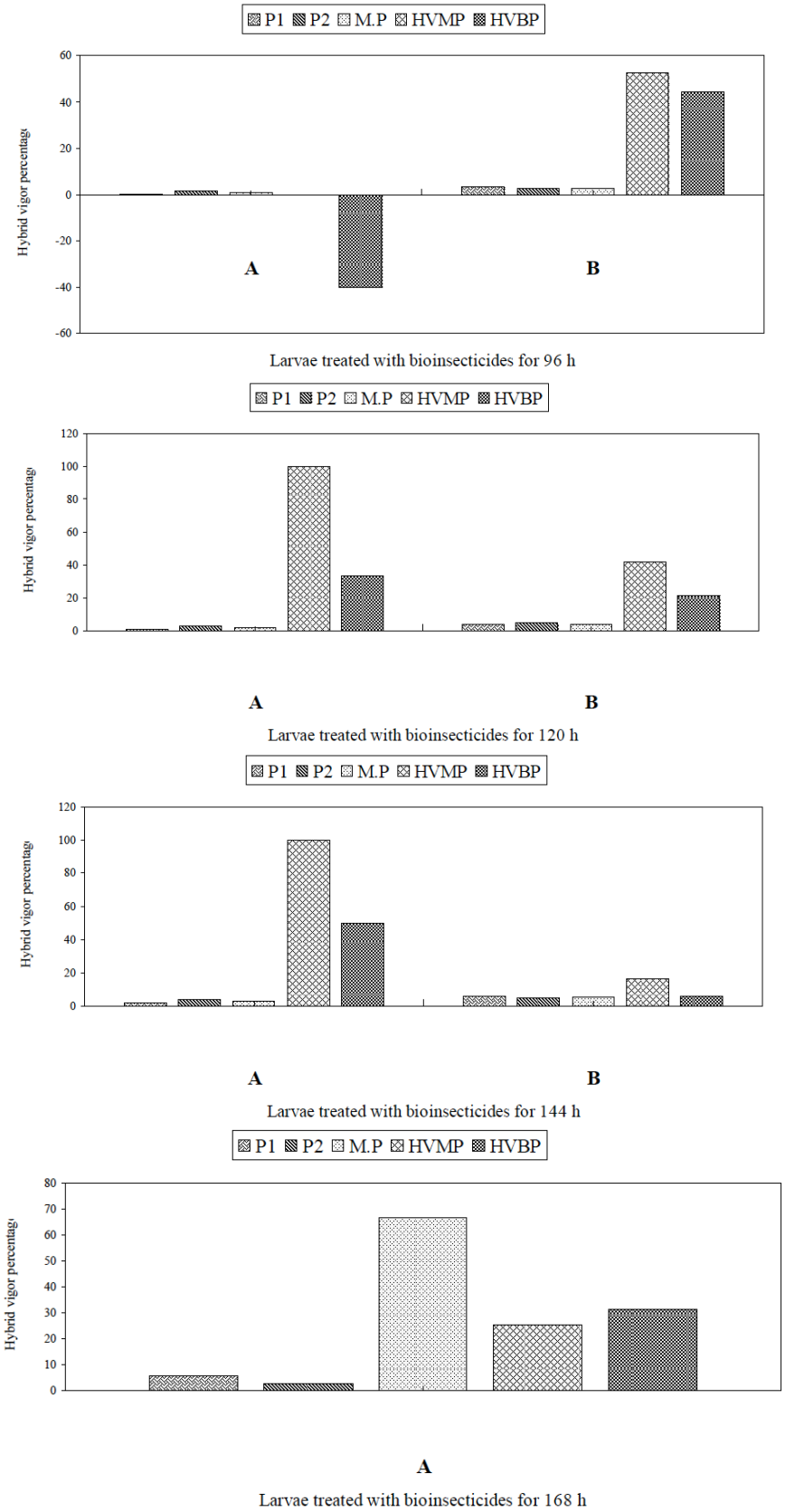


Figure 1: Transconjugant-D.

Figure 1: Continued for transconjugant-D.



Transconjugant-D.

Figure 1: Transconjugant efficiency of recombinant bioinsecticides affecting on reducing larvae survival of *Spodoptera littoralis*. Note: A = Crystals, B = Crystals + Endospores.

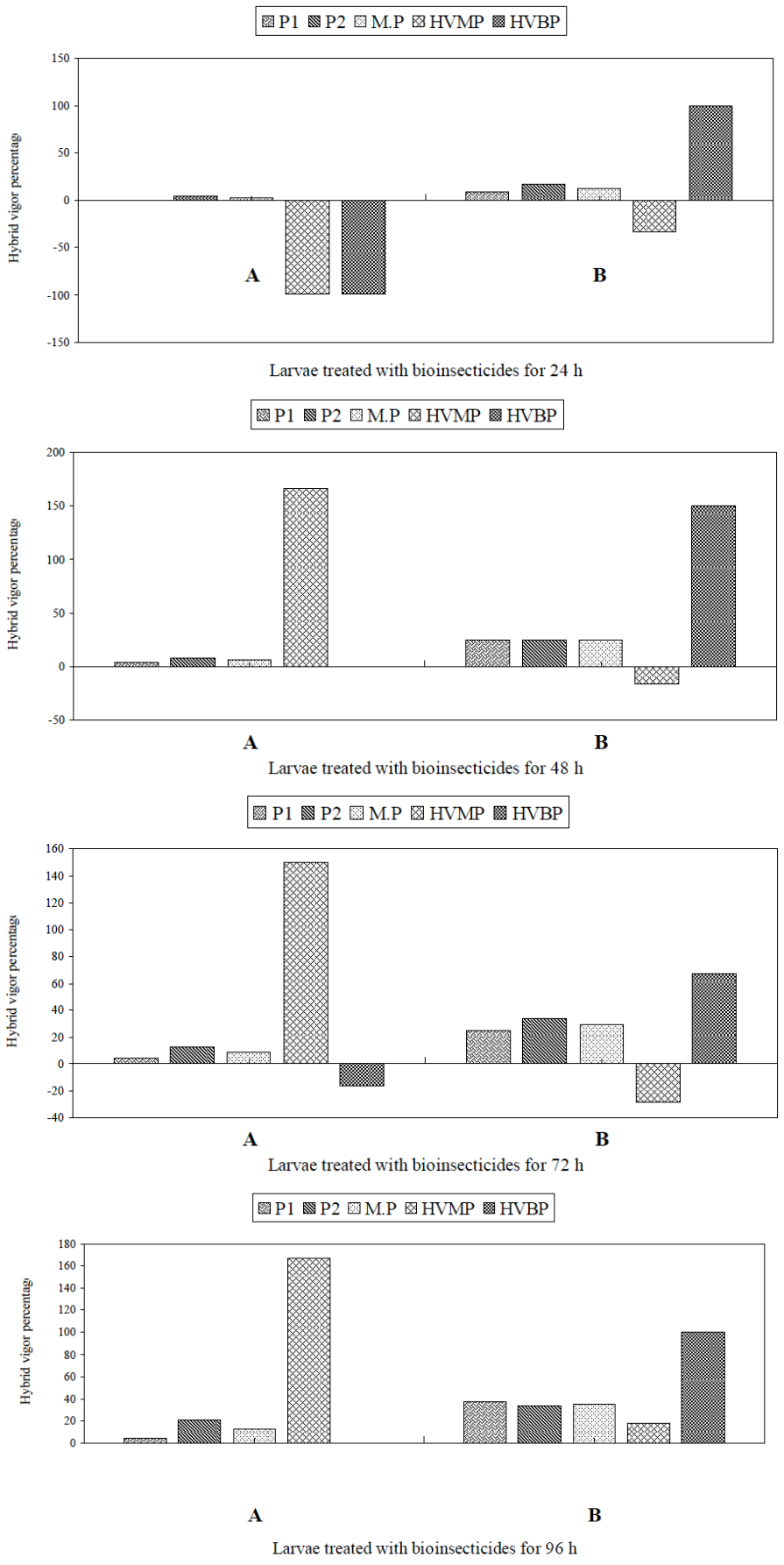


Figure 2: Transconjugant-A.

Figure 2: Continued for transconjugant-A.

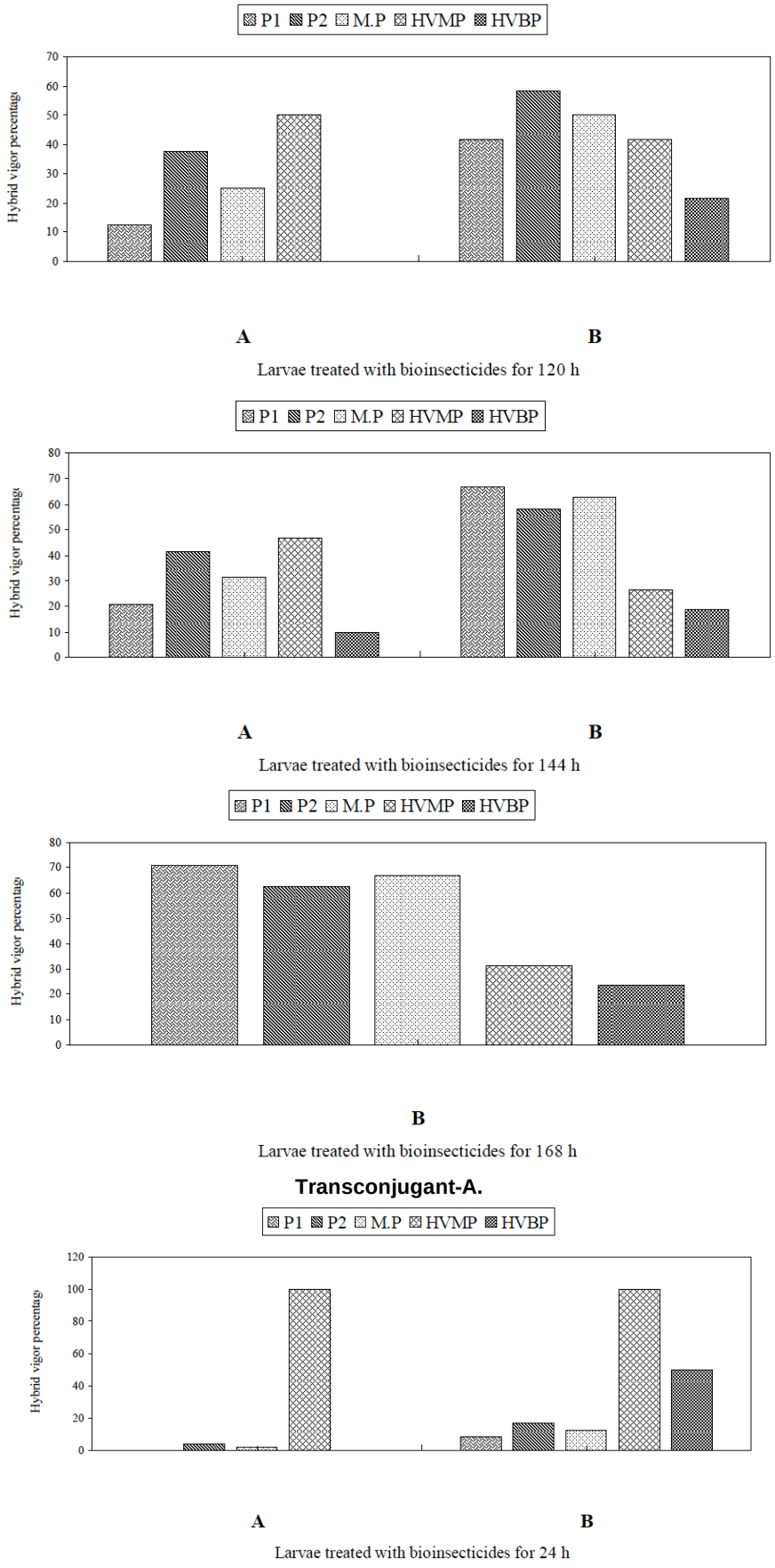


Figure 2: Transconjugant-B.

Figure 2: Continued for transconjugant-B.

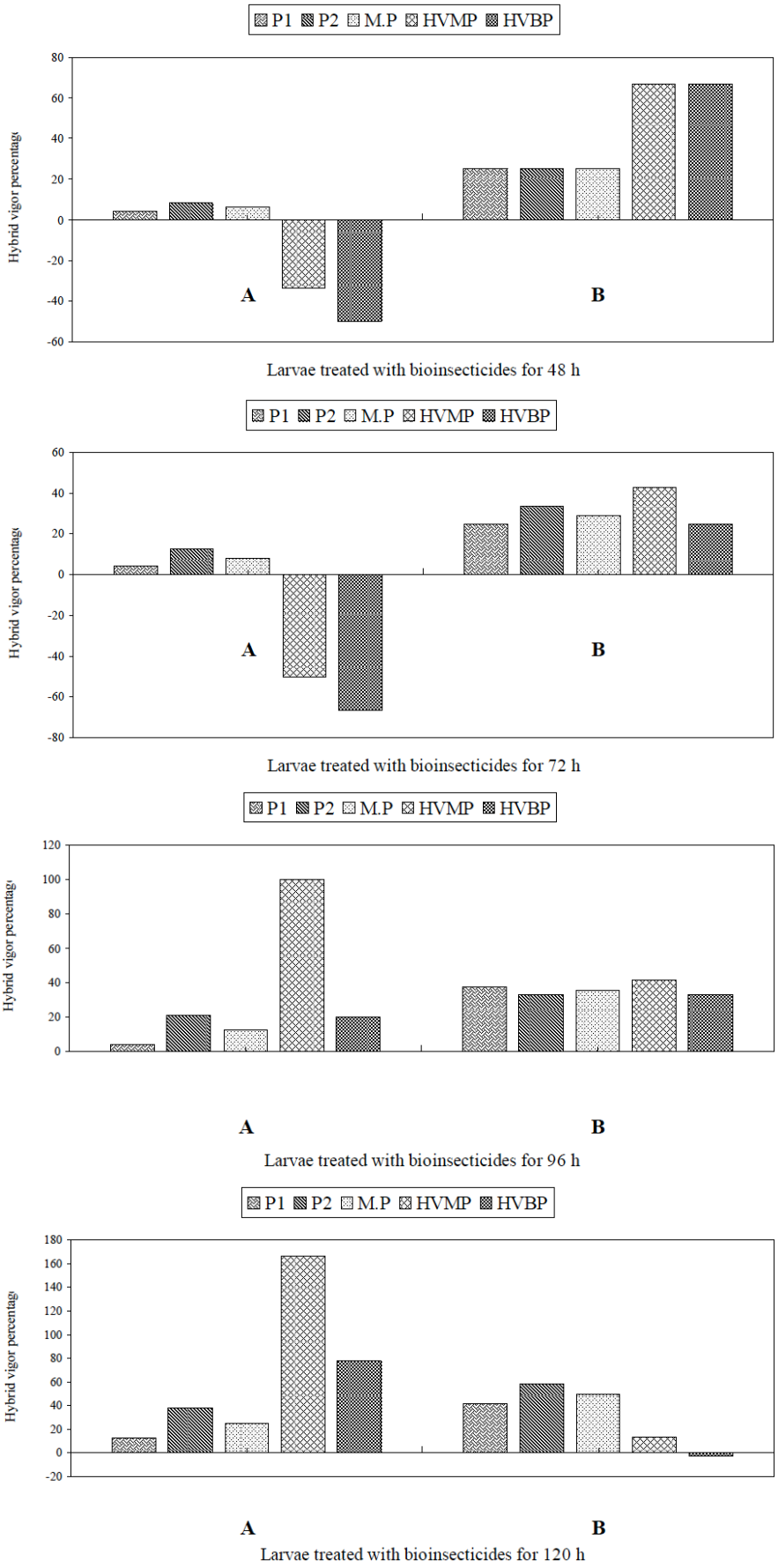


Figure 2: Continued for transconjugant-B.

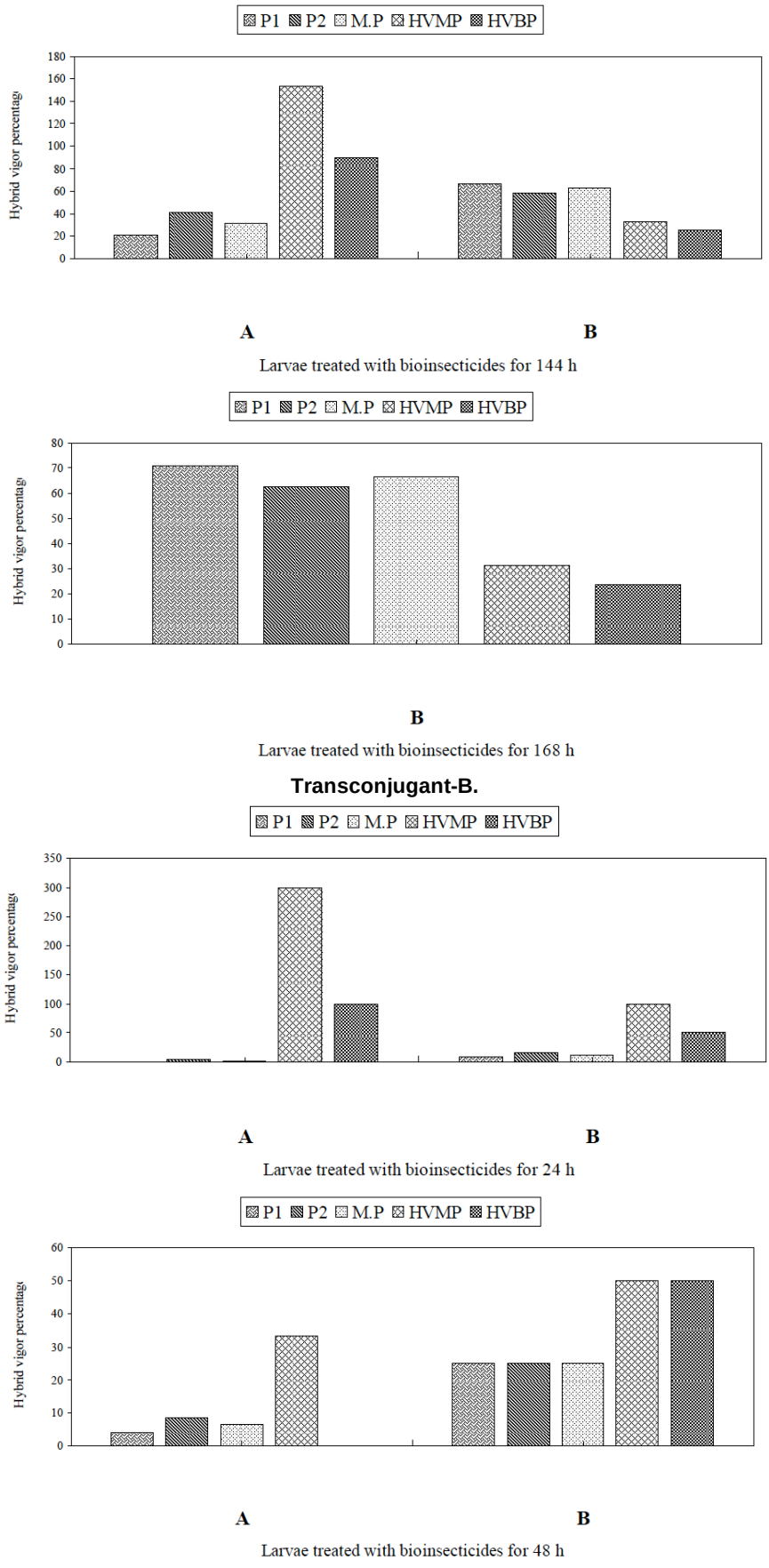


Figure 2: Transconjugant-C.

Figure 2: Continued for transconjugant-C.

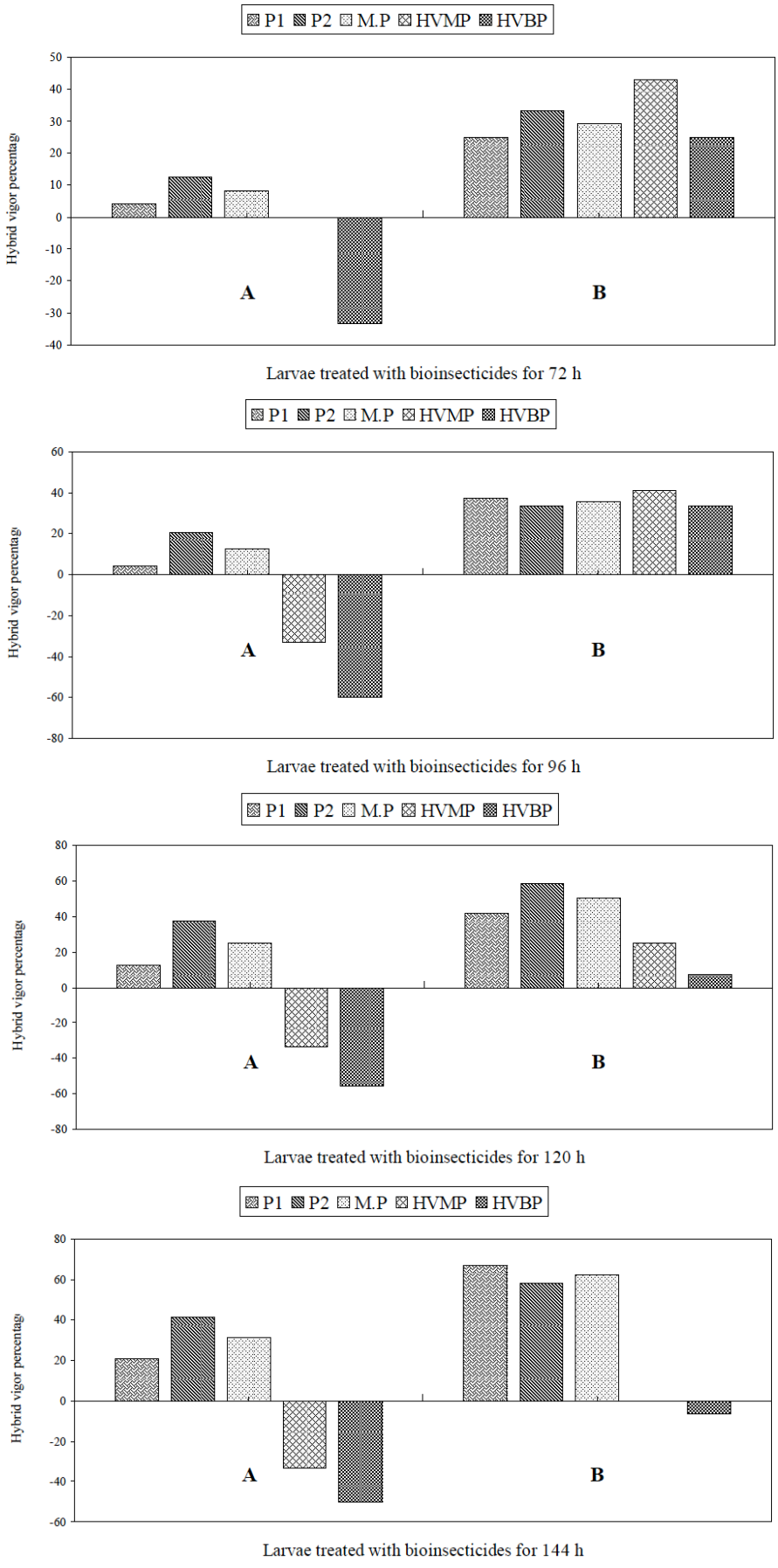
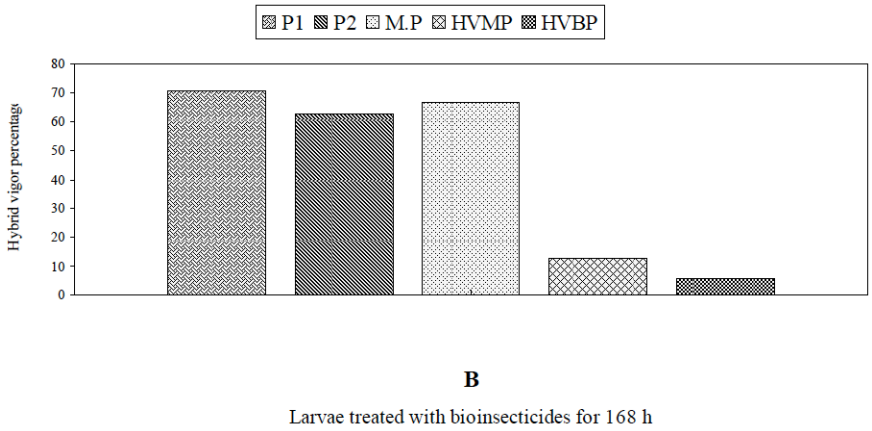
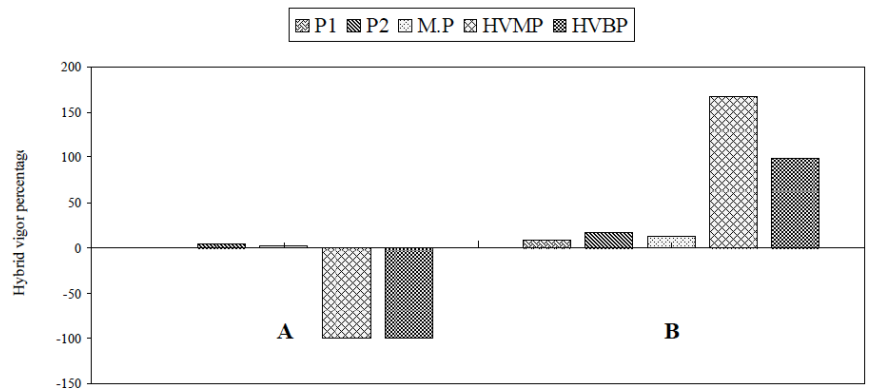


Figure 2: Continued for transconjugant-C.

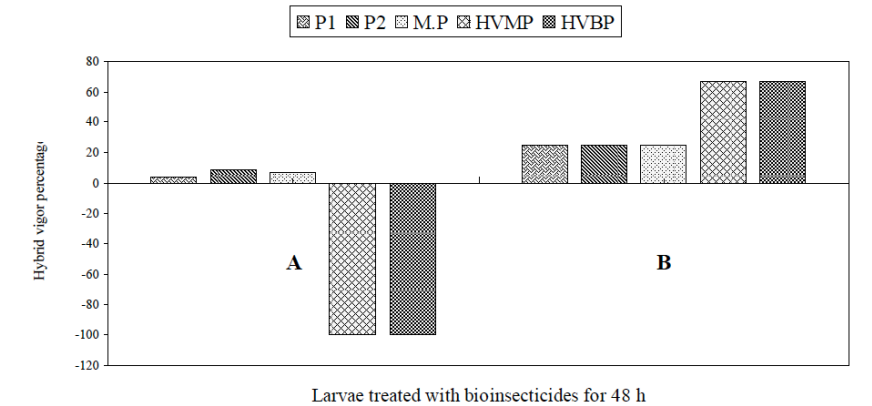


Larvae treated with bioinsecticides for 168 h

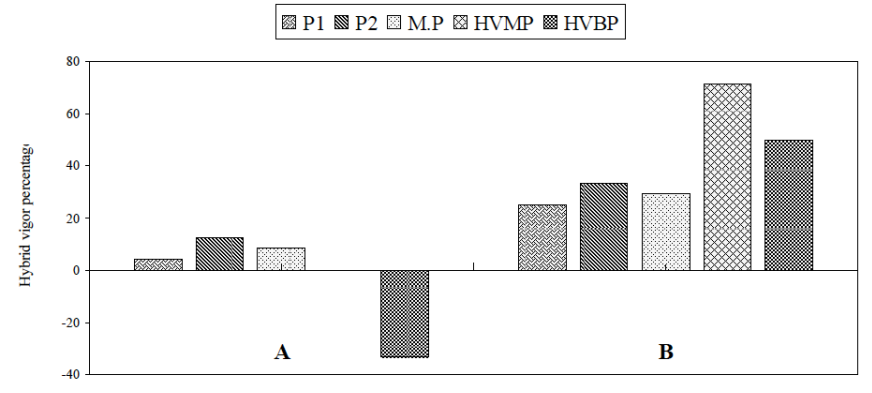
Transconjugant-C.



Larvae treated with bioinsecticides for 24 h



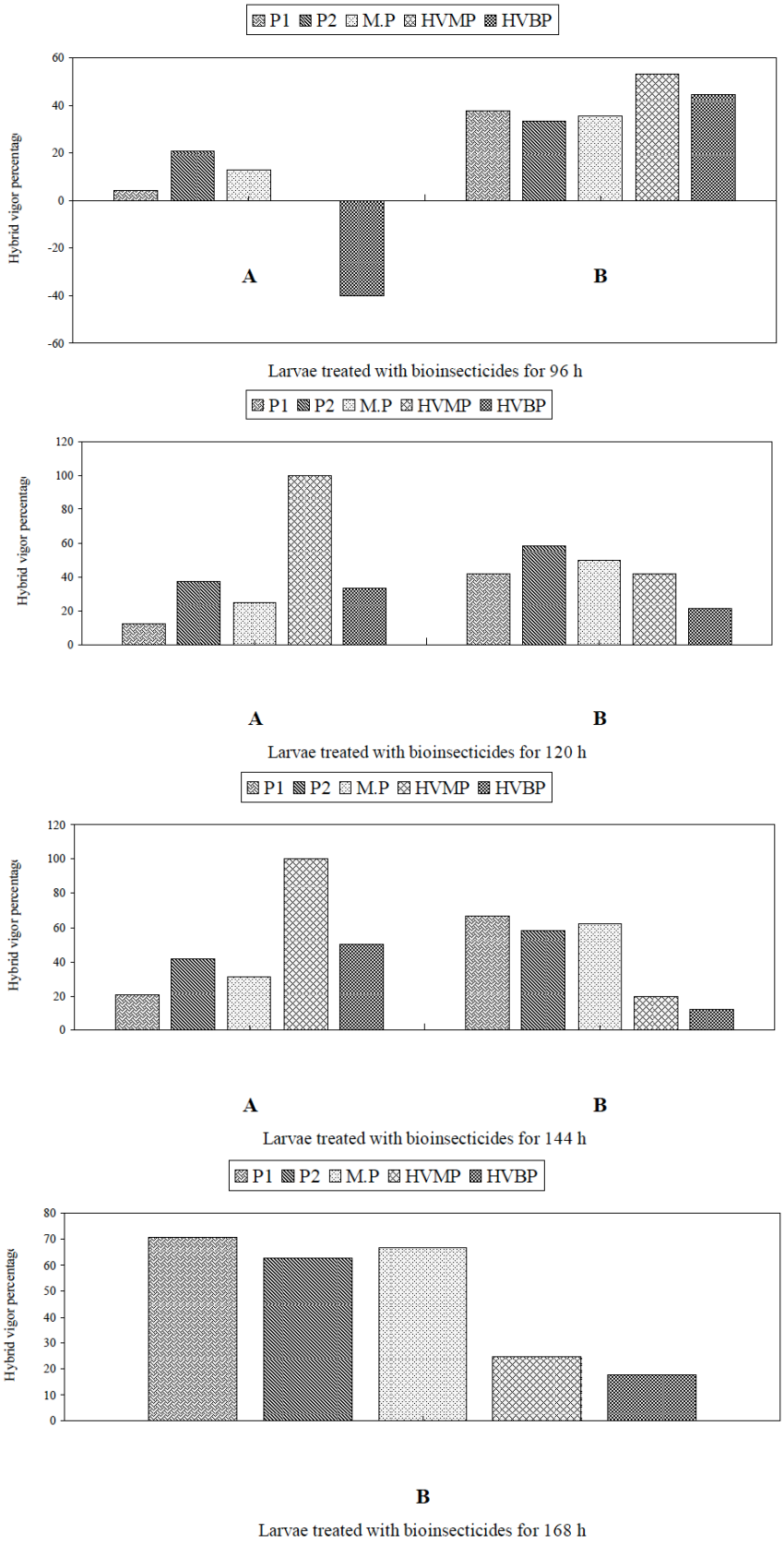
Larvae treated with bioinsecticides for 48 h



Larvae treated with bioinsecticides for 72 h

Figure 2: Transconjugant-D.

Figure 2: Continued for transconjugant-D.



Transconjugant-D.

Figure 2: Transconjugant efficiency of recombinant bioinsecticides in mortality percentage induced in *Spodoptera littoralis* (Transconjugant A).
Note: A = Crystals, B = Crystals + Endospores.

vigor in mortality percentage in relation to the midparents and better parent at 120h and 144h when the larvae was treated with crystals than that treated with crystals + endospores. The results indicated that recombinant bioinsecticides recorded highest mortality than their parents. However, crystals + endospores exhibited higher mortality percentage in most treatments. These results focused on the potential of recominant entomopathogens in biocontrol management of *S. littoralis* larvae.

The results of Zhu *et al.* [31] demonstrated that no significant differences were observed in total survival from hatching to adult, or in larval and pupal durations of *P*. *japonica* supplied with aphids fed on either transgenic or non-transgenic cotton. Similarly, no significant differences in longevity, reproduction, weight, or fatty acid contents of adult beetles were detected. Their results suggested that this type of transgenic cotton might have little effect on the survival, development, and fecundity of *P*. *japonica* through this food chain. Risk assessments and a long history of safe use indicated that *Bt* - crops produce less risk to human health and the environment than do the chemical alternatives. Most available data indicated that *Bt*-transgenic crops have no effects on populations of beneficial predator insects; by contrast, even drifts of chemical sprays clearly affect the abundance of beneficial insects [32].

The construction of such recombinant *Bt* strains has two advantages: (1) the utilization of *Bt* as biopesticide involves the dissemination of large amounts of spores in the environment, and the environmental impact is difficult to assess. Therefore, the presence of viable spores in formulated *Bt* products is not authorized in several countries. Non-sporulating, and consequently non-persisting strains could be used for release into the environment in these countries; (2) one of the limitations of *Bt* products is the rapid inactivation of the crystals in the field. It has been shown that *Bt* toxins encapsulated in *P. jluorescens* cells persist longer in the environment [33]. As crystals produced in the *Bt spoOA* mutant remain encapsulated within the cell, it is anticipated that this should also partly protect them from degradation in the environment. However, the fate of toxins in the environment needs to be better documented.

Using the expression system of *cry* gene and endospores resulted in high level production of this toxin in various *Bt* recombinant strains. This system can thus be used for expressing heterologous proteins of industrial interest in *Bt.* The use of *cry* expression system for production of an additional *cry* protein in a *Bt* strain already harboring one *cry* gene proved to be an effective approach for constructing *Bt* recombinant strains. The quantity of each crystal protein produced in the recombinant strain was not limited by simultaneous production, or competition for sigma factors. Finally, the construction and utilization of a sites specific recombination vector harboring two copies of crystal genes in direct orientation facilitated the construction of recombinant *Bt* strains.

In most lepidopteran species studied, the early instars were more susceptible to *Bt* toxins than later instars. This has been observed for *Ephestia cautella* Walker (Lepidoptera: Phycitidae), *Plodia interpunctella* Hubner (Lepidoptera: Pyralidae) [34]. This phenomenon could be related to size because later instars are larger and therefore able to physiologically tolerate more toxin [35]. It is possible therefore that larval parasitoids could be exposed to Bt toxins *via* spraying their hosts with *Bt*. *Bt* toxins influence the development of a number of lepidopteran species [36], though most of these previous studies have used sublethal toxin concentrations over prolonged periods. In this study the larvae were exposed to Bt for a relatively higher duration. Obonyo *et al.* [22] found that development time of the fifth instar for *C. partellus* larvae subjected to transient feeding on Bt maize at the same growth stage was not affected, possibly because pupation follows shortly after the fifth larval stage in this species at which time the larvae are relatively inactive and do not feed much as they clear up their guts in preparation for pupation [37]. In addition, their large sizes enable them to tolerate more toxin [38]. Overall, larval development time in these larvae was significantly longer as a consequence of *Bt* exposure. This indicates a disturbance to the "normal" development cycle, from which the larvae may eventually recover.

Developmental delays caused by feeding on *Bt* maize and asynchrony of adult emergence could result in susceptible individuals mating before resistant adults emerge, thus potentially weakening this management option [28]. However, there are various alternative stem borer host plants [39], which could be used to delay emergence of susceptible stem borer moths and hence enhance the chances of mating between resistant and susceptible individuals [40].

In conclusion, this work demonstrated that recombinant bioinsecticide of *B. thuringiensis*

containing crystals and endospores appeared more values of hybrid vigor than that containing crystals for increased mortality percentages of *S. littoralis* larvae. This work represents a significant model in improving bacterial bioinsecticides to achieve microbial control of the pests. Field assessments are also required to determine how strong the effect of *Bt* recombinants on cotton leafworm populations, reported here from laboratory experiments, would be development under field conditions.

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