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Efficacy of *Bt* Transgenic Sugar Beet Lines Expressing *cry1Ab* Gene Against *Spodoptera littoralis* Boisd. (Lepidoptera: Noctuidae)

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ABSTRACT

The sugar beet, *Beta vulgaris* L., has some key pests in Iran that causes losses in crops. One of these pests is Egyptian leaf worm, *Spodoptera littoralis* (Boisd). In addition to southern regions of Iran especially in Khuzestan province, this pest is distributed throughout the countries around the Mediterranean region. One of the most effective controlling measures against this pest is planting *Bt* transgenic sugar beet, expressing an insecticidal-protein gene from *Bacillus thuringiensis* subsp. *kurstaki* (Berliner). Larval mortality, larval weight loss, and leaf weight loss as a damage index of this pest on 10 lines of transgenic and 2 non-transgenic sugar beet were studied. In each treatment, 40 larvae of the Egyptian leaf worm (10 larvae/replication) were fed on the leaves of the transgenic lines in glass petri dishes. Larval mortality, weight of the alive larvae, and weight loss of the leaves were recorded after 3 and 6 days. ANOVA results revealed that there were significant differences between the treatments at the 1% probability level considering all evaluated factors. Therefore, mean comparisons were carried out using Duncan's multiple range tests. Based on the results, all of the examined transgenic lines were more effective than non-transgenic line. In addition, the transgenic line S 37-3 was the most effective line among evaluated transgenic lines.

Key words: Spodoptera littoralis, sugar beet, transgenic lines, *Bacillus thuringiensis,* toxins, control.

INTRODUCTION

Sugar beet, *Beta vulgaris* L., is a major industrial crop. The root of this plant has a high concentration of sucrose. Sugar beet is commercially planted to produce sugar. One of the most devastating Lepidopteran pests feeding on sugar beet is *Spodoptera littoralis* (Boisdual). It is a subtropical species of the family Noctuidae and polyphagous insect pest that widely distributed throughout the countries around the Mediterranean basin (Gómez de Aizpurua & Arroyo, 1994). The pest causes severe loss in many outdoor and greenhouse horticultural crops (Bayoumi *et al*.,1998). The Sugar beet and other host crops are damaged by *S. littoralis* in the southern parts of Iran. The larval infestations mainly occur in summer and early autumn, rarely in

winter and spring, causing considerable ecological and economic impacts (Martins, 2000). Control strategies against this pest have basically been focused on pesticide application. Unfortunately, considerably high levels of resistance by *S. littoralis* to many pesticides have been detected over the few years before 1999 (Smagghe *et al*., 1999).

In order to control of Lepidopteran pests, plants have been genetically engineered to express insecticidal proteins from *Bacillus thuringiensis* (*Bt*) subsp. *Kurstaki* (Berliner) (e.g. *Bt*-cotton) and are now planted in the around the world (Torres & Ruberson, 2008). There is also a complex of non-Lepidopteran herbivores that is unaffected by the toxin. Among these unaffected taxa are stink bugs, plant bugs, aphids, whiteflies, mites, and thrips (Torres & Ruberson, 2008). Non-susceptible Lepidopteran and non-Lepidopteran herbivores can survive feeding on *Bt*-plants are capable of conveying toxin to the next trophic level (Obrist *et al*., 2005, 2006; Harwood *et al*., 2005; Torres *et al*., 2006). Insect-resistant transgenic plants with *Bt* toxins genes have been developed in several crops (Hilder & Boulter, 1999; Sharma *et al*., 2000, 2004). Transgenic sugar beet with *Bt* toxins genes has now been developed for controlling beet leafminer, *Pegomya hyoscyami* (Smigocki *et al*., 2003; Wilhite *et al*.,2000).

There is little information on the *Bt* transgenic sugar beet in the world. There is only one report of the development of *Bt*-transgenic sugar beet plants (Kimoto & Shimamoto, 2001). In addition, genetically modified (GM) sugar beet is becoming increasingly interesting as a green bioreactor, i.e., for synthesis and accumulation of new metabolites in roots (Menzel *et al*., 2003). Genetic engineering of crop plants for enhanced resistance to insect pests has been one of the real successes of transgenetic technology during the recent decades (Ferry *et al*., 2006). At present time, the introduction of *Bacillus thuringiensis* δ-endotoxin (*Bt*) genes in crop plants ranks among the most successful strategies for improving tolerance to certain insects (Peferoen, 1997; Romeis *et al*., 2006).

The object of this study was to evaluate 10 *Bt* transgenic sugar beet lines expressing *cry1Ab* gene for controlling of *S. littoralis*. This article reports the results of laboratory bioassays with transgenetic *Beta vulgaris* lines expressing *cry1Ab* and compares the results with bioassays using non-Bt (NBt) *S. littoralis*.

MATERIALS AND METHODS

This study was carried out in Biological Control Research Department (BCRD) of Iranian Research Institute of Plant Protection (IRIPP), during 2008-2009.

Rearing methods

The Laboratory colony of *S. littoralis* used in the study was originally collected in 2008 from unsprayed sugar beet fields, located at the Dezfoul agriculture and natural resource research center, in the south western portion of Iran. Collected larvae were reared on artificial diet (Jafari *et al.,* 2009). Laboratory rearing was conducted at temperature, 25±1˚C with 65±10% relative humidity (RH), and a photoperiod of L:D 16:8 h. Larval rearing containers were cylindrical plastic jars (Behin Plastic Co., Iran) with 10 cm diameter and 4.5 cm in height. The F_2 descendants of the field collected larvae were used for the assay.

Plant material

Two diploid sugar beet genotypes including, multigerm 7233 (S), and monogerm HM1990 (H) provided by the Sugar Beet Seed Institute (SBSI), Karaj, Iran were used as plant materials. These plants were transformed using *Agrobacterium tumefaciens* containing a synthetic *cry1Ab* gene (Hisano *et al*., 2004; Norouzi *et al*., 2005). Transgenic plants were currently grown in a green-house (at temperature, 20±2˚C; 70±20% RH, and a photoperiod of 16:8 h (L:D). Transgenic sugar beet leaves (T_1 generation) were used for testing. Totally, 10 *Bt* transgenic lines as treatments and 2 non-transgenic plant (NBt) types as controls were the source of plant tissue used in these experiments. For testing, fresh, fully expanded leaves were harvested from each line and transported to the laboratory in a cooler containing ice to prevent desiccation.

Design of experiments

Before starting experiments, all glass petri dishes were sterilized inside oven (at temperature 120˚C for 2h). Then, 10 neonate (<24 h-old) larvae were placed on a moistened filter paper in petri dishes (with 9 cm diameter) with separated leaves from T_1 transgenic sugar beets (as treatments) and common planted variety (as control). Larvae were translocated by an artistic brush (size no. o.ooo). Finally, the petri dishes were sealed with parafilm. The assay experiments were done at temperature 25±1˚C, 65±10% RH, and a photoperiod of 16:8 h (L:D) in growth chamber. In the current study, Larval Mortality, Larval Weight Loss (LaWL) and Leaf Weight Loss (LWL) as a damage index on 10 transgenic, and 2 non-transgenic sugar beets lines were examined as 12 treatments in three, separate, completely randomized design (CRD) experiments with four replications in each experiment. Therefore, 40 larvae of the Egyptian leaf worm in four replications (10 larvae/replication) were fed on transgenic leaves of examined lines. In order to estimate the values of considered indices, number of the alive larvae, weight of the alive larvae and weight of the remained leaves were recorded 3 and 6 days after infestation.

Actual values for larval mortality percent were estimated using the following equation (Abbot, 1925).

$$\%Effect = \left(1 - \frac{N_t}{N_c}\right) \times 100$$

where N_t and N_c is the number of alive larvae in treatment and check lines, respectively.

Similarly, the values of the LaWL and the LWL were corrected by subtract the effect of the same index values in check lines from the transgenic lines.

Statistical analysis

Prior to analyses, all of the recorded data were tested for normality by Anderson-Darling normality test, and means were compared by one-way analysis of variance (ANOVA) followed by a Duncan's multiple range test (DMRT) at 0.01 probability level (*P*), when significance was confirmed. All analysis was performed using SPSS software version 13.0 Windows (SPSS Inc., 2003).

RESULTS

Efficacy of the transgenic sugar beet lines were assayed against *S. littoralis*, a polyphagous and important pest in Mediterranean countries and also sugar beet fields of Iran. Based on the obtained results, ANOVA confirmed statistically significant differences at the 1% probability level among treatments in all of the evaluated factors. In the first test, the number of alive larvae on control plants were higher significantly than transgenic lines at 3rd Day After Infections (DAI) (df=9; F=3.184; P=0.009) and 6th DAI (df=9 ; F=2.719; P=0.019). Based on these findings, transgenic sugar beets had higher efficacy against *S. littoralis* than the controls. The range of the percent larval mortality was between 17.5% and 45% in transgenic lines, whereas, it was 2.5% in check line. In the second experiment, none of the larvae that were recovered from the transgenic plants had developed to the next instar. Whereas, in control lines, all of the larvae developed to the next instar and caused considerable damage on leaves. LWL percent was from 59.82±1.17 in 3rd Day After Infection (DAI) to 83.65±2.00 in 6th DAI in control line for multigerm (S) transgenic lines. In addition, LWL percent was from 43.26±0.66 in 3rd DAI to 95.50±1.72 in 6th DAI in control line for monogerm transgenic (H) lines. The mean value of the LaWL fed on *Bt* lines varied from 0.76 to 1.52 mg at 3 DAI (df=9; F=8.418; P<0.001) and from 1.24 to 2.96 mg at 6 DAI (df=9; F=32.707; P<0.001). Whereas, the means of the same index in the non-transgenic check line were 7.7 and 10.2 mg at 3rd and 6th DAI, respectively (Table 1). In the third experiment, statistically higher damage was observed in the check lines due to higher pest feeding. The range of the %LWL values were from 59% to 93% in S-check (non-transgenic, controls for S lines) line and 43% to 95% in H-check (non-transgenic, controls for H lines) line. ANOVA was confirmed that among examined transgenic lines there were statistically significant differences at 3 DAI (df=9; F=84.671; P<0.001) and 6 DAI (df=9; F=42.508; P<0.001), respectively, considering estimated values for %LWL on each line. (Table 1). In contrast, there were low values for %LWL in transgenic *Bt* lines. The range of the values for the %LWL was from 17.8% to 29.5% and 25.00% to 48.3% at 3 and 6 DAI, respectively (Table 1).

There were statistically significant differences between *Bt* and non-Bt lines, considering evaluated factors (LaWL, Larval Mortality and LWL). Generally, all transgenic lines confirmed increased levels of resistance against this pest.

CONCLUSION AND DISCUSSION

Transgenic sugar beet with gene expression of the crystalline insecticidal protein of *B. thuringiensis* can be considered as an effective contributor in pest management program of sugar beet fields in Iran (Jafari *et al*., 2009). In addition, Egyptian leaf worm, *S. littoralis*, is one of the most important pests in sugar beet fields located in the southern parts of Iran (Behdad, 1993). Among the alternatives for controlling this pest, the use of *Bt* transgenic plants has gained attention due to its efficiency, low cost of the pest management programs and no impact on natural enemies (Romeis *et al.,* 2004; Schuler *et al*., 1999; Schuler *et al*., 2001; Schuler *et al*., 2003). Therefore, in order to introduce a sugar beet line with resistance to *S. littoralis*, we evaluated the

efficiency of 10 transgenic sugar beet lines that had been transformed at the SBSI (Sugar Beet Seed Institute) to express a *cry1Ab* gene from *Bacillus thuringiensis* (*Bt*) subsp. *Kurstaki*. Previously, the impact of *Bt* cotton expressing insecticidal proteins from *B. thuringiensis* on the growth and survival of Noctuidae (Lepidoptera) larvae was studied by Stewart *et al.* (2001). Moreover, laboratory and field evaluations of *Bt* transgenic soybean for control of Lepidopteran pests was confirmed (Macrae *et al.,* 2005). All of these studies and other references here in, were emphasized the effectiveness of the *Bt* transgenic plants for control of Lepidopteran pests.

The present study was the first attempt to evaluate the effect of sugar beet *Bt* transgenic lines against *S. littoralis*. Our findings confirmed that the transgenic sugar beet lines containing a *cry1Ab* gene have significantly more efficacy against *S. littoralis* than the check lines, for all things measured. The effect of transgenic sugar beet lines on growth, development and metamorphosis of *S. littoralis* was similar to those were reported for the most studied pest species from the family Noctuidae by Stewart *et al.* (2001), Macrae *et al.* (2005) and Sivasupramaniam *et al.* (2008). In the literature assays in which larvae were fed fresh plant tissue expressing both *Cry1Ac* and *Cry2Ab* were more toxic to bollworms, *Heliciverpa zea* (Boddie), fall armyworms, *Spodoptera frugiperda* (J.E. Smith), and beet armyworms, *Spodoptera exigua* (Hübner), than single-toxin cultivars expressing *Cry1Ac* (Stewart *et al.,* 2001). In addition, the findings of Macrae *et al.* (2005) were revealed that transgenic soybean exhibiting high-dose expression of a synthetic *B. thuriniensis cry1A* gene had complete efficiency against *Anticarsia gemmatalis* Hübner, *Pseudoplusia includens* (Walker), *Epinotia aporema* (Walsingham), *Rachiplusia nu* (Guenee), and *Spilosoma virginica* (F.). In other study by Sivasupramaniam *et al.* (2008), the efficiency of *Cry1Ac* protoxin (the active insecticidal toxin in both Bollgard and Bollgard II cotton [Gassypium hirsutum L.]), and *Cry2Ab2* toxin (the second insecticidal toxin in Bollgard II cotton) were assayed against five of the primary Lepidopteran pests of cotton by using diet incorporation. Results were shown that *Cry1Ac* was the most toxic to *Heliothis virescens* (F.) and *Pectinophora gossypiella* (Saunders) and had a good efficiency against *H. zea* but had negligible toxicity against *S. exigua* and *S. frugiperda*. In contrast, our findings were confirmed that the sugar beet lines expressing *cry1Ab* gene had an acceptable effect against the *S. littoralis*. This conflict may be due to difference between *Cry1Ab* and *Cry1Ac* toxin proteins or pest species. It can be more clarified if following studies confirm the efficiency of transgenic cultivars expressing *Cry1Ab* protein against the other pest species of *Spodoptera* spp.. In addition, Sivasupramaniam *et al.* (2008) were reported that *Cry2Ab2* had the most toxicity to *P. gossypiella* and least toxicity to *S. frugiperda.*

Our findings confirmed between multigerm (S) and monogerm (H) series of transgenic lines, S lines presented more efficiency against the studied pest. Furthermore, S18-12 caused the most larval mortality among S series of studied lines. However, it should be considered that all of the S transgenic lines had no statistically difference at 5% probability level and were located in the same statistical group. On the other hand, examined transgenic sugar beet lines had strong anti-feedant effect on *S. littoralis*. This increased the developmental time and mortality.

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Table 1. Larval mortality percent and larval weight loss of *Spodoptera littoralis* fed on different sugar beet transgenic lines expressing *cry1Ab* gene *Bt* and percent of leaf weight loss due to larval feeding on examined transgenic sugar beet lines.

Line Index	Larval Mortality Means(1) ± SE		Larval Weight Loss (mg) Means ± SE		% Leaf Weight Loss Means ± SE	
	3 DAI (2)	6 DAI	3 DAI	6 DAI	3 DAI	6 DAI
S18-12	22.50±2.50^{a}	45.00±4.78^{a}	0.68±0.03^{c}	11.67±0.06^{cd}	36.90±1.07^{e}	67.37±0.94^{d}
S32-2	7.50±4.78^{bc}	35.00±6.29^{ab}	0.98±0.06^{b}	11.28±0.09^{ef}	38.70±0.70^{ef}	66.98±1.12^{d}
S35-3	17.50±4.78^{ab}	35.00±2.50^{ab}	1.42±0.23^{a}	12.17±0.06^{b}	40.92±0.48^{f}	67.90±1.26^{d}
S36-13	12.50±2.50^{abc}	37.50±4.08^{ab}	0.96±0.06^{bc}	11.39±0.10^{e}	30.30±0.90^{d}	67.00±1.38^{d}
S37-3	15.00±2.88^{abc}	37.50±7.07^{ab}	1.44±0.07^{a}	12.52±0.03^{a}	41.99±0.09^{f}	67.30±2.50^{d}
S38-3	15.00±2.88^{abc}	32.50±2.88^{ab}	0.77±0.02^{bc}	11.16±0.03^{f}	41.09±1.29^{f}	66.80±2.30^{d}
H2-13	15.00±4.78^{abc}	22.50±4.78^{bc}	0.91±0.04^{bc}	11.66±0.16^{cd}	19.22±1.68^{b}	53.88±1.74^{bc}
H2-33	22.50±2.88^{a}	35.00±4.08^{ab}	1.33±0.08^{a}	11.76±0.06^{c}	24.61±1.06^{c}	56.73±1.17^{c}
H3-2	10.00±2.50^{bc}	17.50±2.50^{c}	1.04±0.01^{b}	11.85±0.04^{c}	14.65±1.04^{a}	47.19±2.52^{a}
H6-8	5.62±1.87^{c}	32.50±4.78^{ab}	0.96±0.02^{bc}	11.48±0.04^{de}	18.50±1.38^{b}	50.38±2.33^{ab}

1 - Means in the same column followed by different letters are significantly different at P<5% (Duncan's Multiple Range Test).

2 - DAI=Days After Infestation.

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