

## Environmental Risk Assessment of Watermelon Grafted onto Transgenic Rootstock Resistant to Cucumber Green Mottle Mosaic Virus (CGMMV) on Non-Target Insects in Conventional Agro-Ecosystem

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**ABSTRACT:** We investigated the impact of watermelon grafted onto Cucumber Green Mottle Mosaic Virus (CGMMV)-resistant transgenic watermelon rootstock on insects as non-target organisms in a greenhouse in 2005. We quantitatively collected insect assemblages living on leaves and flowers, and we used sticky traps to collect alate insects. We compared the patterns of insect assemblages and community composition, cotton aphid (*Aphis gossypii* Glover) on watermelon leaves and western flower thrip (*Frankliniella occidentalis* Trybom) on watermelon male flowers, between CGMMV-resistant transgenic watermelon (TR) and non-transgenic watermelon (nTR). Non-parametric multidimensional scaling (NMS) ordination verified that insect assemblages on leaves and sticky traps were different between TR and nTR ( $P < 0.05$ ). The insect assemblages on male flowers were not statistically significant. Multi-response permutation procedures proofed our results from NMS results ( $P > 0.05$ ). Conclusively, TR watermelons appear to have some adverse effects on the population of cotton aphids on leaves and sticky traps, but watermelon male flowers do not show an adverse effect. Further research is required to assess the effect of TR on the aphid and western flower thrip. Life table experiments might support the specific reason for the adverse effects from leaf assemblages. Assessment of non-target impacts is an essential part of the risk assessment of non-target insects for the impact of transgenic organisms.

**Key words:** CGMMV transgenic watermelon, Cotton aphid, Non-target impacts, Western flower thrip

### INTRODUCTION

Genetically modifying plants (i.e. transgenic plants) is a powerful science that allows moving genetic materials intentionally between organisms with the aim of changing their characteristics to improve crop cultivars, such as resistance to certain herbicides, diseases, or pests (Fraley 1992, Simmonds et al. 1999, Griffiths et al. 2000, Conner et al. 2003). The scientific advances in cell and molecular biology, including biotechnology, will continue to develop in genetically modified crops (Conner et al. 2003). Many genetically modified organisms (GMOs) have been developed, and the cultivation areas and commercial markets of genetically modified (GM) crops increase annually around the world (James 2005). Although GM crops may potentially improve the reliability and quality of the world food supply, public and scientific concerns have been raised about the environmental safety and food safety of these crops. The safety of GM human food is controversial because of the possibility of transmittance of foreign materials (Conner et al. 2003, Nap et al.

2003). Therefore, a risk assessment for non-target organisms on all GM crops must be a priority for safe human food, and the application could resolve public and scientific concerns (Conner et al. 2003, Nap et al. 2003, Badosa et al. 2004). The identifying and developing scientific methodologies and tools available for environmental risk assessment (ERA) of meaningful pre-release testing and post-release monitoring and management of transgenic plants are required, in accordance with the Cartagena Protocol on Biosafety and other international agreements (UN DSD 1999, <http://www.gmo-guidelines.info>). The GMO ERA Project is currently in its pioneer stage, driven by public sector scientists that includes experts in environmental science, biotechnology, and socioeconomics fields. The project addresses the environmental and agricultural effects of transgenic crops (Hilbeck and Andow 2004, Hilbeck et al. 2006). The non-target organism effects can be evaluated using experiments designed to identify hazard, determine levels of exposure, and assess if the hazard constitutes a risk (Cowgill and Atkinson 2003). Practically, every non-target organism cannot be applied to assess the potential risks of GM plants. Therefore, the first step of the risk assessment

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process is to select the proper non-target species to investigate the effects on organisms (Bigler and Babendreier 2006).

Common watermelons are monoecious, bearing both male flowers and female flowers. Each flower is approximately 2.5 cm in diameter and is self-fertile. Although watermelon has two pollinators, such as bees and bumble bees (Delaplane and Mayer 2000), an artificial cross method for fertilization is commonly used in conventional agriculture. Rootstock grafting is common in the cultivation of watermelon in South Korea and Japan (Park et al. 2005), but rootstock is vulnerable to virus infections, such as cucumber green mottle mosaic virus (CGMMV), which causes a loss of income for *Curcubitaceae* crop farmers (Park et al. 2005). Therefore, the transgenic watermelon rootstock (*Citrullus lanatus* (Twinsler) cv. Gongdae) developed by Nong Woo Bio Co. (Park et al. 2005) for rootstock grafting could satisfy both the public's concern for transmittance of foreign genes into fruits and the farmers' needs for resistance to diseases in the agro-economy (Conner et al. 2003, Nap et al. 2003).

Because CGMMV is easily transmitted from soil, the development of a virus-resistant rootstock offers a viable solution. Because no genetic source is available for resistance against CGMMV infection yet, Park et al. (2005) used an alternative viral gene, a coat protein (CP) gene, and proved that the CGMMV-resistant TR watermelon rootstock (gongdae) did not transmit mRNA and protein into the leaves and fruits (KRIBB report 2005).

It has been documented that transgenic crops impact agroecosystems and natural ecosystems through direct and indirect ways, including gene flows, invasions, and community and/or food web changes (Dale et al. 2002). Although previous studies on the relationship between insects and their hosts are documented, there is a lack of risk assessment for the relationship between phytophagous insects and their host, transgenic watermelon. Cotton aphids (*Aphis gossypii* Glover) and western flower thrips (*Frankliniella occidentalis*), the major pests of watermelon, are known to vector >50 plant viruses, including CGMMV (Sylvester 1989, Wijkamp 1995, Cranshaw 2004). Our objective was to assess the environmental risks of the effects of transgenic watermelon (TR) compared to non-transgenic watermelon (nTR) with a diverse insect community and the populations of cotton aphids and western flower thrips on watermelon grown in conventional agricultural conditions.

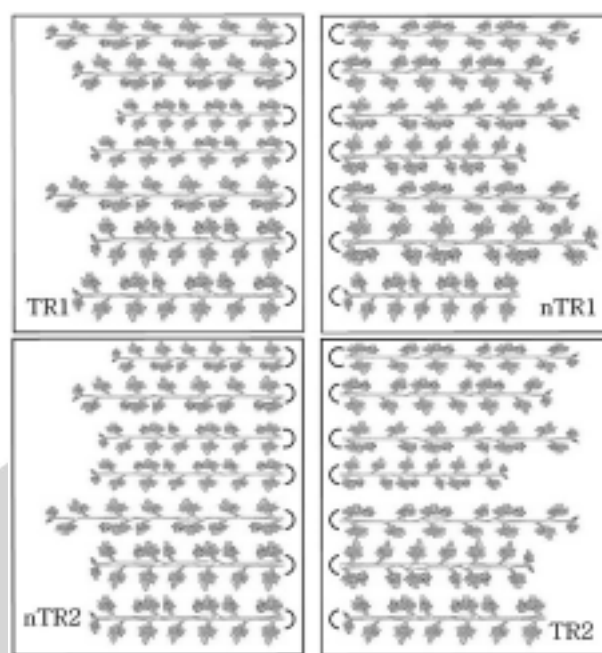
## MATERIALS AND METHODS

### Plant Cultivation and Experimental Designs of Greenhouse Plot Experiment

Watermelons grafted onto the transgenic CGMMV-resistant rootstock (Park et al. 2005) were planted in an isolated plastic green-

house in Miryang Institute managed by Nong Woo Bio Co., according to the experimental design scheme (Fig. 1A).

The plot experiment was conducted in the plastic greenhouse of the Miryang Institute of the Nong Woo Bio Co. in South Korea (E128°47', N35°30'). CGMMV-resistant transgenic (TR) and non-transgenic (nTR) watermelons (*Citrullus vulgaris* [Twinsler] cv. Gongdae) were planted in two replicated plots with 7 watermelon plugs planted under restricted regulation. The total area of the experiment was 5×5m<sup>2</sup>, and the area was divided by 4 plots (Fig. 1).



A. An experimental design



B. A sticky trap

Fig. 1. A. The experimental design of the plastic greenhouse in Miryang where 7 watermelons were planted at each treatment. B. A sticky trap photo from the plastic greenhouse in Miryang (TR: Transgenic watermelon; nTR: non-transgenic watermelon).

## Sampling and Identification of Insects

One week before sampling, we notified the farm managers to not apply pesticides that might affect the population size of fauna. In the event that there might exist residual effects from pesticide, we tried to collect samples in similar conditions between TR and nTR.

### 1) Sampling Insects

#### (1) Leaves

Three leaves from each watermelon plant in each treatment were randomly sampled for insect assemblages on June 24 in 2005.

#### (2) Flowers

We collected only male flowers to avoid disturbing other experiments counting the number of fruit. Consequently, the number of flowers were limited, and female flowers affected the rates. We randomly took only five male flowers from each treatment on June 16, June 24, and July 26 in 2005.

#### (3) Sticky Traps

We hung one two-sided yellow sticky trap (Green Agrotech, Korea. 12.5 cm × 15.0 cm) on a stick (30cm height) at each plot to collect flying insects from June 16 to June 24 in 2005. The traps, which are a non toxic way to monitor flying insects, are patterned in a grid to help precisely count population levels.

### 2) Insect Identification

Samples were stored at the 5°C until insect identification under a dissect microscope was executed to identify the species or genus. A voucher collection is maintained in the LMO lab at Korea Research Institute of Bioscience and Biotechnology.

## Statistical Analyses

Analysis of variance (ANOVA) were used to test the differences between TR and nTR watermelons in species richness, mean species diversity, mean abundance, and total abundance for the insect community with the SAS software package (SAS Institute 2001), and F-statistics were calculated for type of crop, collecting season, and their interactions. In all analyses, the level of significance was at least  $P = 0.05$ .

Ordination analyses were done using PC-ORD version 4.28 (McCune and Mefford 1999, McCune and Grace 2002). The Shannon-Weiner Diversity Index ( $H'$ ) was calculated (McCune and Grace 2002). The pooled main matrices for each insect sample had high  $\beta$  diversity, moderate to extreme row and column skewness, and a high coefficient of variation among the sums of the columns (species) in the matrices. Thus, the data were transformed by taking

logarithms and relativization by column (species) maxima to equalize the weights between abundant and less abundant species. The Sørensen distance measure was used for all multivariate analyses. Non-metric multidimensional scaling (NMS) (Kruskal 1964, Mather 1976, Clarke 1993) is an iterative method based on rank distances between sample units. It is useful for ecological gradient studies because of its general robustness and lack of assumptions about the distribution or type of data. Therefore, NMS was used to determine the number of factors structuring the complex community and to qualitatively summarize the overall distribution of species assemblages across the gradients of different treatments. NMS was used in lieu of other ordination methods because it avoids the zero-truncation problems of Beals (1984). We used multi-response permutation procedures (MRPP), which focus on the analysis for the type of crop alone (McCune and Grace 2002). This procedure was useful for analyzing most of the ecological data because it did not require assumptions of normality and constant variance (Biondini et al. 1988, McCune and Mefford 1999).

## RESULTS

### Insect Communities from the Greenhouse Experiments

To assess the environmental risks of the non-target organisms associated with the transgenic watermelon, we quantitatively sampled the non-target insects using three collecting methods in both the TR and nTR agriculture: leaves, sticky traps, and male flowers. We compared the insect community composition of TR and nTR with species richness, Shannon-Wiener diversity index, mean and total abundances on Table 1. We examined those variables with the different collecting methods in TR and nTR with the ANOVA tests at the 0.05 level.

We collected and identified 11 taxa: *Frankliniella occidentalis* (Trybom) (western flower thrip), *Frankliniella intonsa* (Trybom) (flower thrip), *Aphis gossypii* Glover (cotton aphid), parasitoid wasp, other Hymenoptera spp., Diptera, wooly fly, Cicadellidae, Lygaeidae, Midge, and ants. From leaf samples, we identified 6 taxa: western flower thrip, flower thrip, cotton aphid, other Hymenoptera spp., Diptera, and ants. The dominant species was a cotton aphid. The species richness between TR and nTR respectively was 3.57 and 3.86 ( $F = 0.70$ ,  $P = 0.4095$ ), and the Shannon-Wiener diversity index was 0.31 and 0.21 ( $F = 1.76$ ,  $P = 0.1957$ ). Mean abundance per gram of leaf dry weight was 111.53 and 174.94, and total abundance per gram of leaf dry weight was 669.17 and 1049.64 ( $F = 1.72$ ,  $P = 0.2012$ ) (Table 1). Apparently, the mean abundance and total abundance on leaves seemed to be vastly different but the confident intervals of standard errors were large.

We averaged the three harvests of male flower data. We identi-

fied 3 taxa on flowers: western flower thrip, flower thrip, and cotton aphid. Western flower thrip was the dominant species. From ANOVA, we found that there were seasonal effects for species richness ( $F = 14.56$ ,  $P < 0.0001$ ), Shannon diversity ( $F = 4.51$ ,  $P = 0.0154$ ), mean abundance and total abundance ( $F = 18.55$ ,  $P < 0.001$ ) and there were not interaction effects between types of crops and season for species richness ( $F = 2.67$ ,  $P = 0.0781$ ), Shannon diversity ( $F = 0.88$ ,  $P = 0.4196$ ), mean abundance and total abundance ( $F = 0.09$ ,  $P = 0.9175$ ). Species richness from the watermelon male flower was 1.77 for TR and 1.57 for nTR ( $F = 1.61$ ,  $P = 0.2202$ ); Shannon diversity was 0.17 and 0.17 respectively ( $F = 1.76$ ,  $P = 0.1957$ ); mean abundance was 22.29 and 11.49 respectively, and total abundance was 33.87 and 34.47 ( $F = 0.01$ ,  $P = 0.9265$ ) respectively (Table 1).

We used a standing sticky trap to collect 10 taxa: western flower thrip, flower thrip, cotton aphid, parasitoid wasp, other Hymenoptera spp., Diptera, wooly fly, Cicadellidae, Lygaeidae, and midge. The dominant species was cotton aphid. The species richness was 5.75 for TR and 7.25 for nTR ( $F = 4.91$ ,  $P = 0.0690$ ); Shannon diversity was 0.38 and 0.89 respectively with the statistical difference ( $F = 17.91$ ,  $P = 0.0035$ ); mean abundance was 112.18 and 107.80 respectively, and total abundance was 1234.00 and 1185.75 respectively ( $F = 0.01$ ,  $P = 0.9265$ ) (Table 1).

Overall, species richness and diversity was relatively higher in

the sticky traps than on the leaves and flowers.

### Cotton Aphids

We compared the population size of cotton aphids, one of main pests on leaves, with the watermelon leaves and sticky traps between TR and nTR. We found that the population size of cotton aphids was statistically lower on TR watermelon leaves (548/g of leaf) than on the nTR watermelon leaves (962/g of leaf) (Fig. 2Aa,  $P < 0.05$ ). The population size of cotton aphids for sticky traps was also higher at TR than nTR (Fig. 2Ab,  $P < 0.05$ ).

### Western Flower Thrips

The populations of western flower thrips, one of main pests on flowers, were compared between TR and nTR on male flowers during the three collections, and the population differences were not statistically significant (Fig. 2B,  $P < 0.05$ ).

### Multivariate Analyses

We used non-metric multidimensional scaling (NMS) ordination on each collecting method (Fig. 3). Axis 1 explained 62 % of variance, and axis 2 explained 19 % of variance in watermelon leaves and the total NMS results explained 81 % of variation (Fig. 3A). Final stress was 11.363, and final instability was 0.0000 with 63 iterations. To understand the insect taxonomical relationship and

**Table 1.** The comparison of species richness (S), Shannon-Wiener diversity index (H'), mean abundance ( $\bar{A}$ ), and total abundance ( $\Sigma A$ ) for the three different insect collecting methods: leaves, sticky traps, and male flowers from the transgenic (TR) and non-transgenic (nTR) watermelons in Miryang during the summer of 2005. Mean  $\pm$  SE (standard error)

Collecting ways	Sampling duration	Source	Type of watermelon		F	P
			TR	nTR		
Leaves	June 24 (n=28)	S	3.57 $\pm$ 0.27	3.86 $\pm$ 0.21	0.70	0.4095
		H'	0.31 $\pm$ 0.06	0.21 $\pm$ 0.04	1.76	0.1957
		$\bar{A}$ (No/g Leaf)	111.53 $\pm$ 29.73	174.94 $\pm$ 38.14	1.72	0.2012
		$\Sigma A$ (No/g Leaf)	669.17 $\pm$ 178.41	1,049.64 $\pm$ 228.81	1.72	0.2012
Sticky traps	June 16 ~ June 24 (n=8)	S	5.75 $\pm$ 0.48	7.25 $\pm$ 0.48	4.91	0.0690
		H'	0.38 $\pm$ 0.06	0.89 $\pm$ 0.11	17.91	0.0035*
		$\bar{A}$ (No/Trap)	112.18 $\pm$ 32.94	107.80 $\pm$ 24.94	0.01	0.9189
		$\Sigma A$ (No/Trap)	1,234.00 $\pm$ 362.33	1,185.75 $\pm$ 274.34	0.01	0.9189
Male flowers	June 16 ~ July 26 (n=60)	S	1.77 $\pm$ 0.46	1.57 $\pm$ 0.46	1.61	0.2202
		H'	0.17 $\pm$ 0.11	0.17 $\pm$ 0.11	0.01	0.9158
		$\bar{A}$ (No/Flower)	22.29 $\pm$ 5.44	11.49 $\pm$ 5.69	0.01	0.9265
		$\Sigma A$ (No/Flower)	33.87 $\pm$ 16.33	34.47 $\pm$ 17.06	0.01	0.9265

\*  $P < 0.05$ .

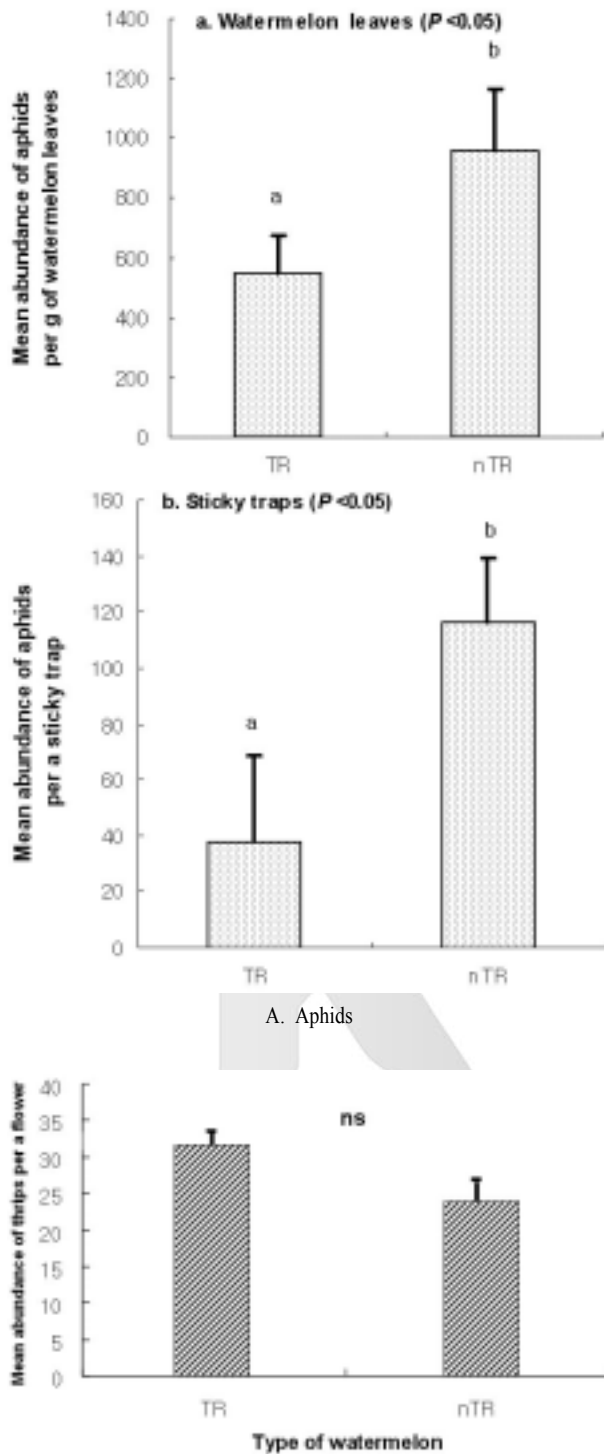


Fig. 2. A. Mean abundance of aphid population from watermelon leaves (a) and sticky traps (b). B. Mean abundance of flower thrip population living on watermelon male flowers. Different characters (a, b) above the standard error bars represent the statistical difference ( $P < 0.05$ ) and ns represents statistically no significant difference ( $P > 0.05$ ).

community composition with the NMS results between TR and nTR watermelon leaves, we examined the Person and Kendall correlations with ordination axes. Cotton aphid (0.80), western flower thrip (0.63), flower thrip (0.46), Diptera (0.60), and ant (0.74) were positively correlated to axis 1; Hymenoptera (0.59) were strongly correlated to axis 2.

In sticky traps, axis 1 explained 40 % of variance; axis 2 explained 35 % of variance; and the NMS results explained 75% of variation (Fig. 3B). Final stress was 4.817, and final instability was 0.0001 with 40 iterations. To understand the insect taxonomical relationship and community composition with the NMS results from the sticky traps, we examined the Person and Kendall correlations. Cotton aphid (-0.90), parasitoid wasp (-0.78), woolly fly (-0.35), and Lygaeidae (-0.55)-except Diptera (0.90)-were negatively correlated to axis 1; western flower thrip (0.63); flower thrip (0.46), midge (0.71), and other Hymenoptera (-0.80) were strongly correlated to axis 2.

The NMS results of watermelon leaves and sticky traps were clearly clustered for TR and nTR. The results of sticky traps were associated with watermelon leaves.

For the male flower community, axis 1 explained 47 % of variance; axis 2 explained 31 % of variance; and the NMS results explained 78% of variation (Fig. 3C). Final stress was 18.61, and final instability was 0.01192 with 500 iterations. The NMS result showed there was not a distinct difference between TR and nTR. To understand the insect taxonomical relationship and community composition with the NMS results, we examined the Person and Kendall correlations. Western flower thrip (-0.76), flower thrip (-0.67), and aphid (0.25) were correlated to axis 1; western flower thrip (-0.45) was correlated to axis 2.

We also used MRPP for the multivariate analyses, and we compared the results on Table 2. The MRPP verified three NMS results (Fig. 3), and we found that there were statistical differences between the watermelon leaf community ( $A = 0.0366$ ,  $P = 0.0271$ ) and sticky-trap community ( $A = 0.1774$ ,  $P = 0.0246$ ). There was not significant difference in the male flower community ( $A = 0.0136$ ,  $P = 0.0698$ ) (Table 2).

**DISCUSSION**

This study was designed to assess the environmental risks at a community level with the biodiversity of non-target insects on grafted transgenic watermelons. The environmental risk analysis, as discussed in Hilbeck and Andows (2004), is used in decision-making processes to reduce adverse effects on the environment, and environmental risk assessment is considered to be comprised of problem identification, hazard and harm identification, effects assess-

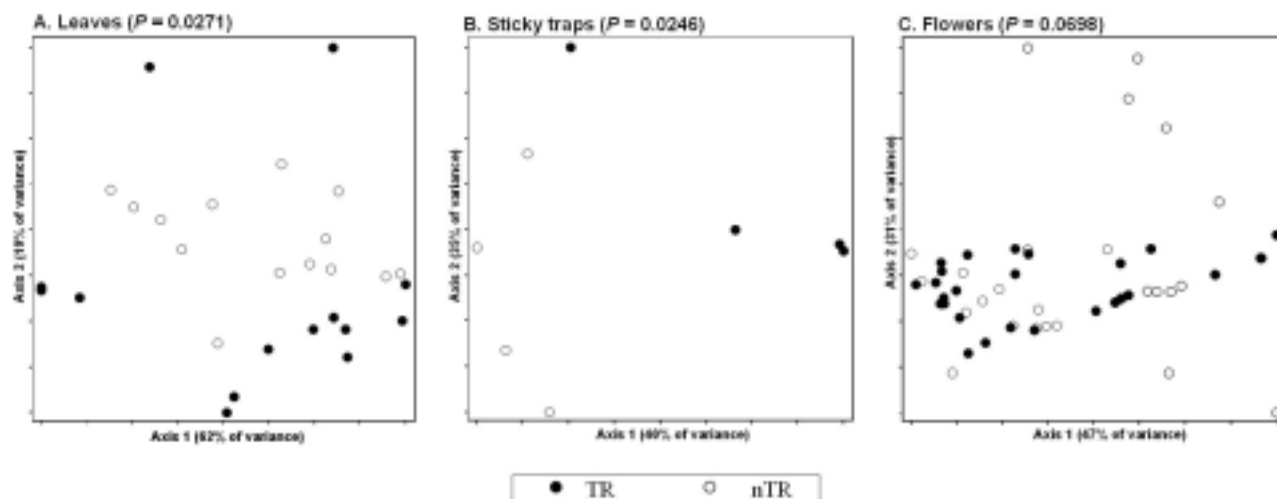


Fig. 3. Non-metric multidimensional scaling (NMS) results between transgenic watermelons (TR) and non-transgenic watermelons (nTR) for the three methods: leaves, sticky traps, and male flowers. A. Leaves (final stress: 11.36; final instability: 0.000; iteration: 63), B. Sticky traps (final stress: 4.82; final instability: 0.000; iteration: 40), C. Male flowers (final stress: 18.61; final instability: 0.012; iteration: 500).

Table 2. MRPP (multi-response permutation procedures) results of watermelons

	Leaves	Sticky traps	Flowers
T	-2.4421	-2.4145	-1.6588
A	0.0366	0.1774	0.0136
P	0.0271	0.0246	0.0698

T = Test statistic, A = Chance-corrected within-group agreement.

ment, and exposure assessment. Our study focused on any adverse effect or difference in community composition by comparing of TR and nTR. Although the few taxa (aphids and thrips) of insects we collected were major pests in most agriculture ecosystems, those species were regarded as non-target species on this transgenic rootstock watermelon. Resistance to CGMMV was the ultimate focus to develop the transgenic rootstock watermelon with insertion of CGMMV-Cp gene (Part et al. 2005)-not resistance to insect pest species on TR watermelon.

We assessed environmental risks with biodiversity and the insect community structure as non-target organisms that consisted mostly of herbivores quantified through various collecting methods on transgenic plants in severely human-controlled environments. Hillbeck et al. (2006) described how biodiversity in an agroecosystem is important to assess environmental risks and guided that most risk assessment for transgenic plants requires a stepwise, case-specific assessment of non-target risks. They also emphasized the importance of the ecological function over taxonomical knowledge of the insect community. When we collected 11 taxa from the greenhouse

in Miryang, we found dominant species, cotton aphids and western flower thrips, that are major pests in greenhouse conditions that incorporate pesticides in conventional agriculture management (Higgins and Mayer 1992, Goh et al. 2002, Park et al. 2002). We caught those species in a greenhouse, but this conventional agro-ecosystem limited the freely accessible non-target insects-such as bees, bumble bees, and flower flies-with the limited screen hole size (less than 0.5 mm). As it is already accepted, greenhouse conditions are suitable for developing two main species throughout the year (Park et al. 2002). When those species are established in a greenhouse, pest control is not easy, but these species are important to assess the environmental risks under a screened experimental condition. In our experiment, we avoided insecticides to create more natural conditions. This is why our mean population of aphids was relatively higher than in Goh et al.'s (2001) experiments.

The population size of cotton aphids on leaves and sticky traps between TR and nTR was clearly different (Fig. 2Aa, Fig. 2Ab), but an indirect explanation could be the difference in leaf size between TR and nTR (personal observation). This was the impetus to measure and compare the population size of aphids with the dry weight of watermelon leaves. The sticky traps were not directly associated to individual plants, but the traps were efficient collecting methods to measure species composition. Specifically, the traps caught alate aphids and other flying insects and show the results of the insect community on leaves. However, the population size of western flower thrips between TR and nTR was not different, and it appears that the more direct effects of watermelon flowers were not different between TR and nTR.

We used non-metric multidimensional scaling (NMS) ordination

on the three different collecting methods, and the NMS results explained the high percentiles of variance at each. Lower final stress (usually less than 20) and lower final instability can improve the confidence levels of the NMS results (McCune and Grace 2002). The data showed well at each graph with the correlation of species scores. The MRPP proofed for the three NMS results (Fig. 3), and it showed *p*-values at each NMS result.

In conclusion, we found that there was a statistical difference for mean abundance of non-target insects, including cotton aphids living on watermelon leaves, but there was not a statistical difference for non-target organisms living on watermelon flowers between TR and nTR watermelons.

We realize that the scale of this experiment was small and limited in access to general non-target insect species; it is a preliminary experiment before going to a large-scale field. We believe that this experiment plays the role of supporting valuable information to assess the environmental risk on transgenic plants with non-target insects. This experiment is ongoing, and further studies in more realistic conditions will confirm if there are any adverse effects of TR watermelons on agro-natural conditions that are accessible by natural non-target insects.

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