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Research article

Invasiveness, allelopathic potential and unintended effects of miraculin transgenic tomato to soil microbes

Nono Carsono[1,*], Fadlilah Aida Rahmani[1], Rangga Jiwa Wibawa[1], Santika Sari[1], Anas[1], Ryo Ohsawa[2], Ayako Shimono[2,3] and Hiroshi Ezura[2]

[1] Lab of Plant Breeding, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor Campus, Sumedang 45363, West Java, Indonesia
[2] Tsukuba Plant Innovation Research Center, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan
[3] Department of Biology, Faculty of Science, Toho University, Funabashi, Chiba, Japan

* **Correspondence:** n.carsono@unpad.ac.id; Tel: +6281395567514.

Abstract: Tomato cv. Moneymaker was modified by the insertion of a miraculin gene, which can modify a sour taste into a sweet taste. Environmental safety assessment for this special transgenic crop is an important step in assessing how safe this tomato is before it is released into the environment. Evaluation of invasiveness, allelopathy and unintended effects is highly essential for environmental safety assessment. The evaluation of invasiveness was carried out by growing a mixture of transgenic and non-transgenic tomatoes with ratios of 0:100 and 100:0 (sole-cultivation) and 25:75, 50:50 and 75:25 (mix-cultivation). Wet and dry biomasses of three-week-old tomato plants were measured. Soil microbes were evaluated by determining microbial populations (culturable) and estimating soil respiration. Microbial populations were determined through total plate count, while soil respiration was estimated using the titration method to calculate the levels of carbon dioxide released during the incubation. It was found that the aggressiveness of the miraculin transgenic tomato was equal to that of its counterpart. There were also no significant differences in microbial populations and soil respiration of miraculin transgenic tomato compared with those of wild type. In addition, miraculin transgenic tomato did not produce allelopathy that interfered with surrounding crops. It is concluded that transgenic tomato is equal to its counterpart in invasiveness, with no effect to soil microbes and no potential allelopathy found.

Keywords: allelopathy; invasiveness; miraculin; soil microbes; transgenic tomato

1. Introduction

The transgenic approach is a promising technique to support food security and improve the quality human life. This technology has high potency to improve production, nutrients, and crop resistance to biotic and abiotic stresses [1]. In 2016, genetically modified crops were planted by farmers in 185.1 million hectares across 26 countries, with 54% of biotech crops in developing countries and 46% in industrial countries [2]. In the United States, as a biotech mega-country, 72.9 million hectares have been growing with commercialized transgenic crops, such as maize (35.05 million hectares), soybean (31.84 million hectares), cotton (3.70 million hectares), alfalfa (1.23 million hectares), canola (0.62 million hectares), sugar beet (0.47 million hectares), papaya (1000 hectares), squash (1000 hectares) and potatoes (2500 hectares) [2].

Sugaya et al. [3] and Sun et al. [4,5] successfully produced transgenic strawberry, lettuce and tomato expressing miraculin gene under the control of a CaMV-35S promoter. The gene is derived from miracle fruit (*Richadella dulcifica*) from West Africa [6]. This transgenic tomato has a potential as an alternative low-calorie food. Miraculin can change a sour taste on the tongue into a sweet taste [7]. Miraculin is not sweet by itself, but if it is inserted into a plant with a sour taste, or we eat miraculin before the sour fruit, the fruit will taste sweet [8]. For example, before enjoying a lemon, if one eats fruits with the glycoprotein miraculin, then the tongue will taste sweetness. When the miraculin is on the tongue, the sweetness level can be over one hour when we feel an acidic solution [9]. The production of miraculin from the original plant (miracle fruit) is limited because it produces fruit when more than four years old [10]. This study suggests to mass production of miraculin because the tomato is convenient to cultivate in tropical countries, such as Indonesia.

The use of transgenic crops can be positive in many aspects, but it also raises concerns about the environment, human health and biodiversity, including miraculin transgenic tomato. Measures to anticipate transgenic crops and their commercialization have been regulated in the laws that apply in each country. The Organization for Economic Cooperation and Development (OECD) in 1993 [11] explained that to assess the biosafety risks of transgenic crops, one can use the concept of substantial equivalence, where the transgenic product is substantially equivalent to the product of non-transgenic origin except in the nature that is engineered [12–14]. Some components of the environmental risk assessment in the Cartagena Protocol, the European Union and countries that grow transgenic crops, such as Argentina, Australia, Canada, China and the USA, are invasiveness, weediness and the impacts of transgenic crops on the soil environment, agricultural cultivation systems and nontarget organisms [15]. The goal of the risk assessment is to identify, characterize and evaluate the safety of cultivation of transgenic plants with respect to health and the environment [16]. Examining the effect of miraculin transgenic tomato plants on microbial soil is important because a quarter of the Earth's total biomass is estimated to be soil microorganisms, especially a group of microflorae such as bacteria, fungi and actinomycetes, which are very dominant populations in the soil. Moreover, the microflora of the soil is a good indicator to determine changes in an ecosystem [17].

Biosafety assessment is one of the requirements that must be met in terms of the release of genetically modified (GM) or transgenic crop varieties. Biosafety assessment testing of miraculin transgenic tomato includes substantial equivalence assessment through invasiveness (environmental safety/biodiversity), allergenicity and toxicity testing (food safety), and it has a very important role for the utilization and release of GM plants, both for human health and the environment. All tests should be conducted with the aim of determining the potential environmental safety and food safety of the genetically engineered crops [18].

Invasiveness testing was done by figuring the biomass value, and this test is very important to determining the safety of miraculin transgenic tomato for the environment or biodiversity. Miraculin tomato also must be assessed for unintended effects in terms of miraculin transgenic tomato that would possibly affect soil microorganisms. Unintended effect testing on non-pathogenic bacteria is beneficial, and it should be conducted to determine the impact of the miraculin tomato on the environment around the planting site [19]. Kusano et al. [10] stated that multi-platform analysis is needed to assess the substantial equivalence of tomatoes over-expressing the taste-modifying protein miraculin.

In this study, an environmental safety assessment for transgenic miraculin tomato was conducted for invasiveness in biosafety containment (BC) and confined field trials (CFT), unintended effects on soil microbes (limited to culturable microbial populations) and potential allelopathy. Until now, there has been no study conducted for this very special tomato in a tropical country like Indonesia. Our purpose was to ensure that transgenic miraculin tomato is safe.

2. Materials and methods

The experiments were conducted in biosafety containment, ICABIOGRAD, Bogor, and a confined field trial at the Experimental Station of the Faculty of Agriculture, Universitas Padjadjaran, Jatinangor Campus. Evaluation for invasiveness was performed according to a method developed for canola [20]. The experiment was arranged in a randomized block design with five treatments (two treatments of monoculture and three treatments of mixture). Various compositions of non-transgenic and transgenic tomatoes were tested, i.e., 0:100 and 100:0 (sole-culture) and 25:75, 50:50 and 75:25 (mix-culture). Wet and dry biomasses of three-week-old tomato plants after planting were measured using relative yield [21]. For drying, tomato plants were stored in a dryer at 55 ℃ for six days.

Relative yield was calculated as follows [20]:

$$ra = {}^{X_{ab}}/_{X_{aa}}, \tag{1}$$

$$rb = {}^{X_{ba}}/_{X_{bb}}, \tag{2}$$

where ra = relative yield of non-transgenic tomato, rb = relative yield of transgenic tomato, Xab = biomass of non-transgenic (mix-cultivation), Xba = biomass of transgenic (mix-cultivation), Xaa = biomass of non-transgenic (sole-cultivation), Xbb = biomass of transgenic (sole-cultivation).

$$Relative\ yield\ total\ (RYT) = ra + rb, \tag{3}$$

$$Aggressiveness\ (A) = (ra - rb)/RYT, \tag{4}$$

where A = 0 = equal competitiveness, A = (+) = high competitiveness, A = (−) = low competitiveness.

The soil respiration and microflora population experiments were conducted at the Laboratory of Biology and Biotechnology of Soil Science Department, Faculty of Agriculture, Universitas

Padjadjaran. The experiment was arranged in a randomized block design with two treatments and repeated four times. They were arranged in zig-zag formation with 70 cm × 50 cm per plant. Both soil samples were evaluated at the times before planting, maximum vegetative period and after harvesting.

Soil respiration was estimated using the titration method to calculate the levels of carbon dioxide released during the incubation of the growing medium. About 100 grams of growing medium and 10 ml KOH solution were incubated in stoppered glass for 7 days. After that, the KOH solution was titrated by HCl 1N solution. Finally, the content of CO_2 from respiration activity was estimated. Meanwhile, microbial populations were determined through total plate count method with Nutrient Agar (NA) medium for Bacteria, Potato Dextrose Agar medium for fungi and Dextrose Nitrate Agar medium for Actinomycetes. Incubation was performed at 25 ℃ for 2 days for bacteria, 5 days for fungi and 10 days for actinomycetes. Data for soil respiration and microflora population in the rhizospheres of transgenic tomato plants and non-transgenic cv. Moneymaker plants were then compared using Student's t-test at a 95% confidence level.

The evaluation of potential allelopathy was done with a sandwich method developed by Fujii et al. [22]. Transgenic and non-transgenic leaves and *Ageratum conyzoides* leaves were put in the same Petri dish. These leaves were placed between layers of agar on 6-well micro-plates, and lettuce seeds were used as seed tests on the surface of the agar. Data for allelopathy were measured by calculating seed germination and the seedling growth of lettuce. Analysis of variance (ANOVA) was performed for these data, followed by Tukey's test.

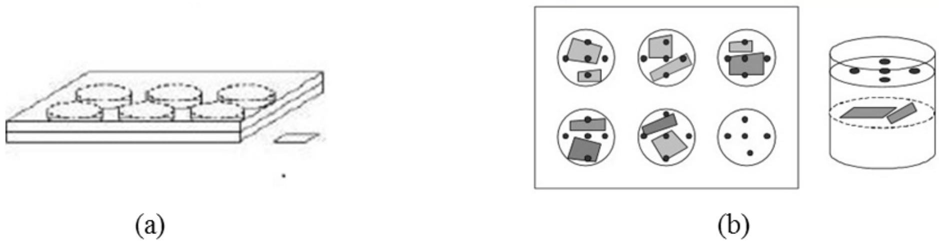

Figure 1. Evaluation of allelopathy through sandwich method. (a): 6-well micro-plate; (b): leaves placed between agar layers. (Source: Fujii et al., 2004)

3. Results and discussion

3.1. Invasiveness

The results of the invasiveness evaluation in biosafety containment can be seen in Table 1 and Table 2, while the results for the confined field trial can be seen in Table 3 and Table 4. Based on Table 1 and Table 2, transgenic tomato cv. Moneymaker showed aggressiveness values of −0.17 in mixture and 0 in monoculture. Table 3 and Table 4 show aggressiveness values of −0.006 in mixture and 0 in monoculture. Meanwhile, wet weight of tomato plants in biosafety containment and confined field trial showed biomass values of −0.10 (Table 1) and −0.012 (Table 3), respectively. In dry weight, biomass was −0.19, and aggressiveness was −0.32 in mixture and 0 (zero) in monoculture (Table 2) in biosafety containment. Meanwhile, in the confined field trial, biomass was −0.015, and aggressiveness was −0.057 in mixture and 0 (zero) in monoculture (Table 4).

The invasiveness values were obtained from tomato plants grown in biosafety containment (BC) and the confined field trials (CFT). Both biomass and aggressiveness for wet and dry weight showed values that were all below or close to zero, indicating that transgenic tomato plants do not have the potential to become invasive, or cultivation of transgenic tomato is safe even if they grow in the mixture. Results on the invasiveness of this transgenic miraculin tomato were quite the same as those reported by Sugaya et al. [3] for transgenic miraculin strawberry.

Table 1. Relative yield and aggressiveness values for mix and sole cultivation (wet weight) of tomato plants in biosafety containment.

Seed composition	Shoot wet weight of non-transgenic (g)	Shoot wet weight of transgenic (g)	Relative yield of non-transgenic	Relative yield of transgenic	Relative yield total	Aggress-iveness
100NT:0TR	312.13	0.00	1.00	0.00	1.00	0.00
75NT:25TR	191.93	86.13	0.61	0.33	0.94	−0.33
50NT:50TR	134.13	159.63	0.43	0.60	1.03	−0.17
25NT:75TR	98.38	244.50	0.32	0.92	1.24	−0.01
0NT:100TR	0.00	264.88	0.00	1.00	1.00	0.00
Biomass		−0.10				
Aggressiveness	Mix-cultivation	−0.17				
	Sole-cultivation	0.00				

Notes: Ra = relative yield of non-transgenic tomato; Rb = relative yield of transgenic tomato; RYT = relative yield total; A = value of aggressiveness. Biomass = average of all aggressiveness values; Aggressiveness in mixture = average value of the aggressiveness of seed composition in mixture; Aggressiveness in monoculture = average value of the aggressiveness of seed composition in monoculture.

Table 2. Relative yield and aggressiveness values for mixture and monoculture (dry weight) of tomato plants in biosafety containment.

Seed composition	Shoot dry weight of non-transgenic (g)	Shoot dry weight of transgenic (g)	Relative yield of non-transgenic	Relative yield of transgenic	Relative yield total	Aggress-iveness
100NT:0TR	15.50	00.00	1.00	0.00	1.00	0.00
75NT:25TR	6.50	07.10	0.42	0.51	0.93	−0.76
50NT:50TR	9.68	10.83	0.63	0.78	1.40	−0.11
25NT:75TR	5.60	15.70	0.36	1.13	1.49	−0.10
0NT:100TR	0.00	13.93	0.00	1.00	1.00	0.00
Biomass		−0.19				
Aggressiveness	Mix-cultivation	−0.32				
	Sole-cultivation	0.00				

Notes: Ra = relative yield of non-transgenic tomato; Rb = relative yield of transgenic tomato; RYT = relative yield total; A = value of aggressiveness. Biomass = average of all aggressiveness values; Aggressiveness in mixture = average value of the aggressiveness of seed composition in mixture; Aggressiveness in monoculture = average value of the aggressiveness of seed composition in monoculture.

Table 3. Relative yield and aggressiveness values for mixture and monoculture (wet weight) of tomato plants in the confined field trial.

Seed composition	Shoot wet weight of non-transgenic (g)	Shoot wet weight of transgenic (g)	Relative yield of non-transgenic	Relative yield of transgenic	Relative yield total	Aggress-iveness
100NT:0TR	65.73	0.00	1.00	0.00	1.00	0.00
75NT:25TR	59.45	21.94	0.91	0.34	1.25	0.00
50NT:50TR	13.41	14.68	0.63	0.20	0.23	0.43
25NT:75TR	22.52	57.64	0.34	0.90	1.25	−0.00
0NT:100TR	0.00	63.85	0.00	1.00	1.00	0.00
Biomass		−0.01				
Aggressiveness	Mix-cultivation	−0.06				
	Sole-cultivation	0.00				

Notes: Ra = relative yield of non-transgenic tomato; Rb = relative yield of transgenic tomato; RYT = relative yield total; A = value of aggressiveness. Biomass = average of all aggressiveness values; Aggressiveness in mixture = average value of the aggressiveness of seed composition in mixture; Aggressiveness in monoculture = average value of the aggressiveness of seed composition in monoculture.

Table 4. Relative yield and aggressiveness values for mixture and monoculture (dry weight) of tomato plants in the confined field trial.

Seed composition	Shoot dry weight of non-transgenic (g)	Shoot dry weight of transgenic (g)	Relative yield of non-transgenic	Relative yield of transgenic	Relative yield total	Aggress-iveness
100NT:0TR	7.63	0.00	1.00	0.00	1.00	0.00
75NT:25TR	7.06	3.20	0.93	0.43	1.36	−0.01
50NT:50TR	2.59	2.83	0.34	0.38	0.72	−0.06
25NT:75TR	3.21	6.97	0.42	0.94	1.36	−0.01
0NT:100TR	0.00	7.42	0.00	1.00	1.00	0.00
Biomass		−0.02				
Aggressiveness	Mix-cultivation	−0.06				
	Sole-cultivation	0.00				

Notes: Ra = relative yield of non-transgenic tomato; Rb = relative yield of transgenic tomato; RYT = relative yield total; A = value of aggressiveness. Biomass = average of all aggressiveness values; Aggressiveness in mixture = average value of the aggressiveness of seed composition in mixture; Aggressiveness in monoculture = average value of the aggressiveness of seed composition in monoculture.

(a) (b)

Figure 2. Transgenic (a) and non-transgenic (b) miraculin plants at three weeks after sowing.

(a) (b)

Figure 3. Transgenic miraculin (a) and non-transgenic (b) tomatoes.

3.2. Effect on soil microbes

The effect of transgenic tomato plants on soil microbial populations (culturable microbial populations) was examined through the observation of bacteria, fungi and actinomycetes, as well as soil respiration, and compared with the control, tomato cv. Moneymaker. It has been known that microflorae such as bacteria, fungi and actinomycetes are very common in the soil. Measurement of soil respiration is one of the important indexes in determining the total microbial activity in the soil [17]. The observations of populations of bacteria, fungi and actinomycetes, soil pH and soil respiration can be seen in Table 5.

The highest populations of microbes in the rhizospheres of tomato plants, either transgenic or non-transgenic, at the relevant time of observation were those of bacteria, actinomycetes and fungi (Table 5). The high populations of bacteria and actinomycetes were observed presumably because the pH of the growing medium was suitable for their growth. The planting medium used in the experiment has a neutral pH (Table 5).

Bacteria generally grow well at pH 7, with actinomycetes at pH values between 6.5 and 8.0 [17]. Meanwhile, the populations of fungi that we observed were smaller than those of actinomycetes and bacteria because the fungi are more suited to living in soil that is acidic [17]. Table 5 tells us about the microbial population's dynamics, which are closely related to the differences in root exudates produced by the roots for plant growth [23]. These exudates act as a source of nutrients and a carbon source for microbial growth [24]. Increased populations of bacteria, fungi actinomycetes occurred at the maximum vegetative period, and then the populations decreased during harvesting time. This condition might be due to the production of root exudates at the highest level and richness in organic acids and proteins [24]. Root exudate production declines with the aging process of plant roots [23]. Decreased production of root exudates also means reduced availability of food in the rhizosphere, causing the populations of bacteria, fungi and actinomycetes to experience famine and death.

In this study, observations of the microbial populations were positively in line with soil respiration. Increasing the microbial population also increases the activity of soil respiration. The observations of soil respiration can be seen in Table 5. The observations of bacteria, fungi and actinomycetes populations and the measurements of soil respiration in rhizospheres of transgenic tomato plants showed no significant differences compared with non-transgenic tomato plants according to Student's t-test at the level of 95%. This condition indicates that the miraculin gene that was inserted into the tomato plants has no effect on the culturable microbial populations and soil respiration, although miraculin protein can be secreted by the roots of transgenic tomato plants, as reported by Hirai et al. [25].

Table 5. Comparisons of population of bacteria, population of fungi, population of actinomycetes, pH of growing medium and soil respiration in the rhizospheres of transgenic tomato plants and non-transgenic tomato plants.

Soil sample from rhizosphere	Before planting	Maximum vegetative period	After harvesting
	The population of bacteria (× 10^9 CFU/gram soil)		
Transgenic tomato plant	6.62 a	7.80 a	4.09 a
Non-transgenic tomato plant	7.38 a	7.84 a	4.15 a
	Population of fungi (× 10^4 CFU/gram soil)		
Transgenic tomato plant	4.79 a	7.05 a	3.88 a
Non-transgenic tomato plant	5.79 a	6.60 a	3.61 a
	The population of actinomycetes (× 10^8 CFU/gram soil)		
Transgenic tomato plant	1.39 a	1.86 a	1.10 a
Non-transgenic tomato plant	1.41 a	2.18 a	1.08 a
	pH		
Transgenic tomato plant	6.94	7.07	7.18
Non-transgenic tomato plant	7.03	7.10	7.19
	Soil respiration (mg CO_2-C/kg/day)		
Transgenic tomato plant	6.43 a	15.00 a	9.00 a
Non-transgenic tomato plant	6.86 a	14.57 a	8.57 a

Note: Values with the same letter in the same column are not significantly different according to Student's t-test.

3.3. Potential allelopathy existence

The transgenic plant can become an undesirable plant in subsequent cultivation. However, being a weed is the result of the actions of many genes. Most crop varieties have been sufficiently domesticated to be unable to survive in the unmanaged field. It is unlikely that a single gene transfer would make a wild plant [26]. Some genes controlling traits inserted by gene transfer may result in a weedy plant that improves fitness. Therefore, it can be destructive to native species. Weeds of various crops compete with cultivated plants for growth factors. In some plants, such as *Brassica napus*, *Brassica rapa* and *Helianthus annuus*, that have some characteristics of weeds, their transgenic and novel traits can make the plants themselves weedy and invasive [27].

In the experiment of potential allelopathy with a sandwich method, there were no significant differences among the control, transgenic and non-transgenic treatments. In contrast, the treatment of *Ageratum conyzoides* was significantly different from the other treatments in terms of seed germination and the seedling growth of lettuce (Table 6).

Table 6. Evaluation of the allelopathic potential for lettuce germination.

Treatment	Leaf weight	Germination percentage (%)	Root and shoot length (mm)
Control (lettuce)	0 mg	73.33 a	7.58 a
Non-transgenic	10 mg	66.67 a	5.64 a
	50 mg	66.67 a	5.30 a
Transgenic	10 mg	66.67 a	4.75 a
	50 mg	53.33 a	5.22 a
Ageratum conyzoides	10 mg	33.33 b	2.83 b

Note: Values with the same letter in the same column are not significantly different according to Tukey's test.

Kuiper et al. [28] explained that if a novel food or its component is found substantially equivalent to an existing food or its component, it can be treated in the same manner concerning safety. Substantial equivalence aims to measure whether the transgenic crop is safe, as compared with its counterpart. The transgenic product and its counterpart must have the same environmental conditions to avoid genotypic and phenotypic differences not related to the genetic transformation process [29].

It is presumed that this evaluation result has no allelopathic effects that endanger other crops, as seen from the lettuce germination, which can occur appropriately. The sandwich method principle is to wipe chemical compounds from leaves placed between two layers of agar media on a 6-well micro-plate, with the seed test placed on the surface of the agar layer [22]. Usually, lettuce is a seed test because it is susceptible to the effects of allelopathy [30]. This finding indicates that transgenic miraculin did not produce allelopathic compounds that interfere with surrounding crops.

4. Conclusions

Environmental safety assessment of miraculin in a limited facility and a confined field trial has been performed for invasiveness, soil microbes and allelopathic effects. Invasiveness testing showed that both biomass and aggressiveness in biosafety containment and confined field trial for wet weight and dry weight of transgenic miraculin tomato with non-transgenic miraculin tomato cultivar Moneymaker were negative and near zero. There were also no significant differences in microflora population and soil respiration of transgenic miraculin tomatoes compared with those of wild type. No allelopathic effects were found, as proved by a sandwich method. In conclusion, the results indicate that transgenic miraculin tomato cv. Moneymaker is substantially equivalent to the non-transgenic tomato except for the presence of the miraculin gene.

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Conflict of interest

The authors declare no conflict of interest.

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