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Application of Chemical Mutagenesis to Increase the Resistance of Tomato to *Orobanche ramosa* L.

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Abstract

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In this study chemical mutagenesis of tomato seeds with Ethylmethanesulfonate was used to obtain lines with increased resistance to *Orobanche ramosa* L. The M2 progeny of the mutant tomato plants was screened for the response to broomrape attack in large scale experiment conducted in greenhouse. Sixteen non-infested by the parasite tomato plants were selected. Their offspring was a subject of another screening for broomrape resistance using an artificial polyethylene bags system. As a result of the experiments six lines with significantly increased level of resistance to *Orobanche ramosa* L., were selected.

Key words: *Orobanche,* mutagenesis, tomato, broomrape control

Introduction

Broomrape (*Orobanche* spp.) is wide spread parasitic weed which affects crops and a number of dicotyledonous species. Broomrape causes significant losses in tomato, tobacco and sunflower production in many countries in the Mediterranean region, Eastern Europe and Russia (Parker and Riches, 1993). Main characteristic of this species is the big number of seeds produced by a single plant and their long period of viability. This makes the control of the parasite in agricultural areas extremely difficult. So far, known methods for broomrape control like herbicides, resistant varieties, trap crops, soil solarization, and biological control have their limitations. That is why the modern understanding for the parasite management is the use of integrated control strategy by combining several of the known methods. Essential part of this strategy is the use of resistant to broomrape varieties (Sackston, 1992; Ruso et al., 1996; Sukno et al., 1999).

Development of broomrape resistance in the crops depends on the biology of the crop and the existence of natural sources for resistance in close related wild species. The best results are reached in sunflower by the creation of cultivars highly resistant to broomrape (Pustovoit, 1976;

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Petrov, 1968; Vranceanu et al., 1980; Parker and Riches, 1993; Melero-Vara et al., 1989). Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops in Bulgaria grown in field and greenhouse conditions.

Tomato cultivation is a main source of agricultural income and has vast economic impact in many rural regions. As it is known, *O. ramosa* causes severe damage to the tomato yield (Díaz et al., 2006, Parker and Riches, 1993), and is a serious threat for the tomato production. Because of the fast development of the agricultural sector in Bulgaria in the last few years, the tomato areas are growing rapidly. The presence of broomrape in the soil can be a limiting condition for the tomato production or be the cause of large economic losses. So far there is no reliable source of broomrape resistance in *Solanaceae* family found in the nature, which makes it impossible to add this trait with close related or inter-specific hybridization (Jacobson, 1986). This implies the need to look for alternative methods to change the existing varieties so that they will have such resistance.

Well known method used with success in plant breading is induced mutagenesis. The application of induced mutagenesis has brought a lot of benefits in the modern agricultural production as à method for crops improvement and addition of new valuable traits into the existing varieties (Ahloowalia et al., 2001).

Ethylmethanesulfonate (EMS) is commonly used mutation agents in the plant science because of its height level of effectiveness. EMS belongs to the group of alkylating agents which are well known mutation inducers, causing point mutations (like C-to-T changes) as well as loss of chromosome segments or deletions (Alcantara et al., 1996). A large number of mutations in plants and cultivars have been achieved by the use of EMS, e.g. resistance to herbicides (Jander et al., 2003) and male sterility (van der Veen et al., 1968). In tomato, EMS showed very high efficiency by inducing different morphological and functional mutations (Watanabe et al., 2007).

The aim of this study is to create tomato lines with increased level of resistance to broomrape by the use of induced chemical mutagenesis. Development of tomato mutant lines resistant to broomrape gives a possibility for effective control and reduction in the use of chemicals which is in accord with the contemporary tendencies for safe and environmental friendly agriculture. Such lines can be used directly in the production or for donors for broomrape resistance in classical selection.

Materials and Methods

Plant Materials

Tomato (*Lycopersicon esculentum* Mill.) seeds from Bulgarian cultivar Bella were provided from IZK Maritsa. It is a direct determinant cultivar suitable for field production with very good qualities for the processing industry. Seeds were stored in refrigerator at 4°C.

Seeds from branched broomrape (*O. ramosa* L.) were collected from infected tobacco plants in South-West of Bulgaria in the region of Gotse Delchev town. Fully-matured stems of the parasite were collected from the tobacco fields and dried in shadow. The seeds were separate by sieves and stored at 4°C.

Chemical agents

Ethylmethanesulfonate (EMS). EMS was purchased from Sigma, USA.

EMS treatment

Tomato seeds from Bulgarian cultivar Bella were the subject to EMS mutagenesis. The EMS concentration was defined based on a lethal dose in which 50% of the seeds lose their germination ability. Before the EMS treatment the tomato seeds passed through a preconditioning period. They were placed in Petri dishes on wet filter paper for 4 days at 4°C, and then dried for 16 hours at room temperature. After the precondition period the tomato seeds were incubated in 200 ml solution of distilled water containing 1.5% EMS in a laboratory bottle by gently shaking. Mutagenesis takes place in a fume hood. The time of treatment was 4 hours. After the time elapsed the laboratory bottle containing the seeds was placed under water and rinsed for 15 min. The seeds were dried on a filter paper and after this sowed by hand in a greenhouse to obtain the seedlings. All the consumables in connection to EMS were put into 1M NaOH as a decontamination solution. At the same time another 100 seeds were used as a control and passed through the same procedures excluding the EMS solution treatment.

Planting M1

Seedlings from the EMS treated seeds were sown in the field and were grown until the fruit ripening period. After that 3 fruits from each M1 plant were picked to obtain the M2 seeds. The seeds were taken out of the fruits, washed with water and left to dry at room temperature for 72 hours, and then placed into paper bags at 4°C for storage.

Test for resistance to Orobanche ramosa L.

Experimental screening of M2 progeny of tomatoes for branched broomrape (*Orobanche ramosa* L.) resistance was conducted under greenhouse conditions. Special tins (2/1 m) placed in a greenhouse were loaded with soil mixed with broomrape seeds (50 $mg.kg^{-1}$ soil). The soil was taken from the field in 50 kg sacks and was autoclaved at 121°C for 30 min. Then it was placed in the tins and broomrape seeds were added. After the soil was carefully mixed, it was distributed in 10 cm layer. The seeds were placed in rows with 10 cm distance between the rows and 5 cm between the plants. In every tin there was one row with control plants. After the first broomrape plants emerged above the soil, a selection of non-infested plants was conducted. Tomato plans were pulled out and their roots were examined for the presence of attached developing broomrapes. Those plants clean from broomrapes were sown in the pots and were used for collecting M3 seeds.

Artificial screening system with polyethylene bags (PEB)

Polyethylene bags system for screening tomato plants for resistance to *O. ramosa* was performed according to Parker and Dixon (1983), with the M3 progeny of M2 plants selected from the greenhouse experiment. This method enables observations on every stage of broomrape development which helps to determine the level and nature of the resistance. Six seeds of each tomato plant were sown in soil with perlite in small pots until the third real leaf emerged. Then they were taken out and after removing the roots were placed in water for several days until new roots started to appear. Following, they were carefully placed in polyethylene bags on fiberglass filter paper GF/A, moistened with sterile water and cov-

ered with constant thin layer of surface sterilized broomrape seeds. After the plant was placed on the paper, the bags were closed carefully and placed in the black box using metal hanger and covered with transparent plastic box in order to keep high moisture inside during the first days. They were placed in the greenhouse at 24°C at day and 18°C at night in photoperiod 16/8 hour. The plants were sprayed with water two times per day during the first three days. On the first day into the polyethylene bags, 10 ml of Hohland (1950) nutrient solution was added. After 3 days the box was uncovered and left opened until the end of the experiment. During the experiment proper amount (10ml) of nutrient solution was sustained. The presence of germinating broomrape seeds was observed and the number of formed tubercles was counted every 7 days.

Statistical analysis was done in the environment of SPPS for Windows (SPSS 11.0, 2004, SPSS Inc. Chicago, Illinois 60606, USA.), by applying one way ANOVA ($P < 0.05$).

Results and Discussion

EMS treatment

In the design of the EMS mutagenesis the desirable number of seedlings after the EMS treatment on level of mortality LD 50% was 3000. After examination of the germination rate of the tomato seeds from cultivar Bella, which showed 86% viability, the starting number was defined at 7000 seeds. The EMS mutagenesis was performed in 2004. After the treatment of the 7000 tomato seed with EMS, 2529 M1 seedlings were generated. The observed mortality was 58%. The number of non-germinated seeds was a mark that during the EMS treatment there was an effective mutagenesis process which caused the dead of more then half of the seeds. The effectiveness of the mutagenesis is essential for obtaining a big number of non-lethal changes in the genome of the survived tomatoes giving the phenotypic expression in the plants.

Assuming that the recessive mutation can not be monitored in the plants received from the mutagenized seeds, screening for broomrape resistance was performed in the next year, when mutations are in homozygous condition, so that they can cause phenotypic expressions. Therefore the M1 plans were planted in the field solely for the purpose of obtaining M2 seeds. The fruits from each M1 plant were picked separately in order to generate families that will be screened for broomrape resistance.

Greenhouse screening of M2 plants for resistance to Orobanche

The screening for broomrape resistance was carried out in the next year under greenhouse conditions. From M1 plants 2467 families were obtained after the EMS treatment. From every family, 20 seeds were taken for the examination and the total number of the screened M2 plants was 49340. All of them were grown under the same conditions (Figure 1). The survey for the presence of developing parasitic plants on the tomato root system was made after the first broomrape shoots emerged above the soil surface. Different development stages of broomrape – tubercles and shoots were observed. All of the control plants had broomrapes attached to their root which was a sign that the level of *Orobanche* infection was enough and gives good conditions for se-

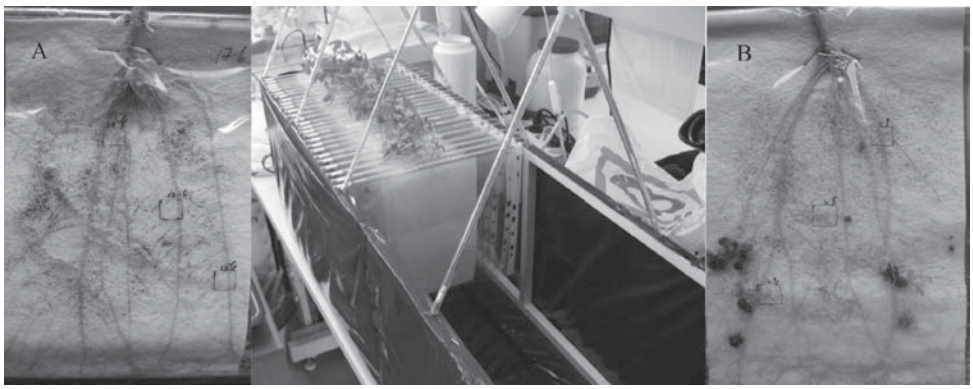
A - tomato with *Orobanche* parasitic plant
B - non-infested with broomrape tomato mutant plant

Fig. 1. Screening of M2 tomato plants for resistance to *Orobanche ramose* L.

lection of potentially resistant mutant plants. The majority of M2 plants was severely attacked by the parasite and also confirmed the effectiveness of the broomrape infection of the soil and the feasibility of the selection process. Different numbers of *Orobanche* plants attached to the root system of the screened M2 plans were present and the variation was from 1 to 25 tubercles on one plant (Figure 1). Only those plants without presence of the parasitic plants on the roots were selected for further examination. None of the M2 families was found to consist entirely of non-infested plants. Single plants with no presence of developing parasites on their roots were selected and transferred in soil again for giving an offspring. Thus seeds from 16 perspective *Orobanche* free plants were collected.

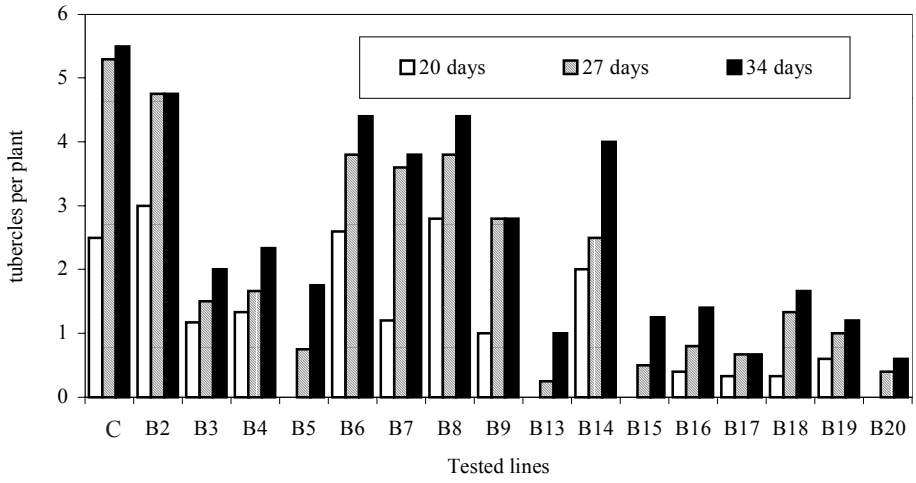
PEB screening of M3

The examination for *Orobanche* resistance of M3 generation was performed via PEB (Figure 2). The presence of germinating broomrape seeds and the formation of tubercles was observed 20 days after the beginning of the experiment. Four lines - B5, B13, B15 and B20 showed no presents of developing tubercles on the first examination while in the control plants there were already 2.5 tubercles per plant (TPP) (Figure 3). The level of broomrape development on these lines till the end of the experiment was significantly lower in comparison to the control plants and some of the other tested M3 lines. The highest number of developed tubercles was 15 and was observed in one of the control plants followed by 11 in line B6. The average number of developed tubercles in the control plants at the end of the experiment was 5.5. Significant difference ($P < 0.05$) was observed in the number of TPP between the control and some of the M3 plants counted 34 days after inoculation. Lines B20 and B17 had less than 1 TPP, respectively 0.6 and 0.7, line B13 had 1 tubercle, while some of the other tested M3 plans had no significant difference with the number of TPP in the control plants – number B2 had 4.8, B6 and B8 had 4.4



A - non-infested with broomrape tomato plant
B - tomato plant with developed broomrape tubercles

Fig. 2. Polyethylene bags screening system for resistance to *Orobanche ramosa* L.



C- control
B2, B3, B4, B5, B6, B7, B8, B9, B13, B14, B15, B16, B17, B18, B19, B20 – tested tomato mutant lines
20, 27 and 34 days after the beginning of the experiment

Fig. 3. Broomrape development in PEB system

TPP. From the sixteen tested M3 lines, six showed significant difference in the number of developed parasite tubercles in comparison to the control plants.

The number of non-infested plants was counted and used for selection of M3 plants (Table 1). Eleven tested lines had at least 1 non-infested plant, as lines B16

Table 1

Brromrape infestation in PEB of M3 selected tomato mutant lines

Lines	C	B2	B3	B4	B5	B6	B7	B8	B9	B13	B14	B15	B16	B17	B18	B19	B20
Number of non infested plants	0	1	1	0	1	0	0	0	0	2	1	2	3	3	1	2	2
Infested with broomrape plants	6	5	5	6	5	6	6	6	6	4	5	4	3	3	5	4	4

and B17 had 3 non-infested plants from all the 6 tested plants. No resistant tomato plant was found between the controls and the lowest number of broomrape tubercles that showed among them was 1 TPP. All the non-infested plants were selected to be a subject of further investigation even those which derived from lines that have shown lower levels of resistance to broomrape. For example, there was one non-infested plant from B2 line which showed 4.8 TPP, the highest level of broomrape development among the tested M3 lines.

After combining the two factors - average number of tubercles per plant and the number of non-infested plants, the most perspective plants were selected. Six lines – B13, B15, B16, B17, B19 and B20 showed significantly lower level of broomrape infestation in comparison to the control and some of the other tested lines.

Considering the results from conducted experiments we concluded that the increase of the resistance to *Orobanche ramosa* L. in this tomato lines is due to genetic change as a result of the EMS treatment. Further investigations, field experiments and molecular studies will give more information about the level and the nature of this resistance. Encheva and colleagues (2003) reported that the use of different methods for mutagenesis - embryo culture, gamma radiation and ultrasound helped to accelerate resistance to *Orobanche cumana* in sunflower. In our study we aim to achieve the increase of the resistance level to *Oroban-che ramosa* using chemical mutagenesis. Possibilities for obtaining tobacco plants resistant to *Orobanche ramosa* using EMS were discussed by Slavov et al. (2001).

Using similar technique in our experiments, we confirmed that this method is also useful for obtaining tomato lines with increased level of broomrape resistance.

Conclusions

The application of EMS for chemical mutagenesis of tomato, followed by a test of the mutant plants for the response to *Orobanche ramosa* L. infestation, lead to the selection of lines with lower susceptibility to the parasite attack in comparison to the original cultivar. The obtained lines can be used as a source for resistance to broomrape in tomato breeding programs.

The obtained results confirm that chemical mutagenesis gives a real possibility for increasing the resistance of tomato to broomrape. Since there is no natural resistance to the parasite, this method could be a very good possibility for limiting the damage done to tomato by this important parasitic plant.

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