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In Vitro Selection of Tomato Resistance to Metribuzin Herbicide. Indices of Tissue Culture and Growth Parameters

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THE EFFECT of metribuzin at different doses (0.25, 0.5, 0.75 and 1.0 kg ai ha^{-1}) was studied on intact seedlings of two tomato variants (*Lycopersicon esculentum* Mill, cv. Karnak vfnt F-1 and Castle rock). The herbicide provoked significant inhibitions in the different growth parameters studied (shoot height, root length, fresh weight and dry weight) of the two tomato variants. The magnitude of inhibition augmented with increasing metribuzin dose. Therefore effectiveness of cyclical *in vitro* selection for metribuzin tolerance in both variants was studied. Cotyledons and leaf explants from shoot regenerated on media containing different concentrations of metribuzin (0, 0.63, 1.25, 2.5, 5 and 10 µM) were used to initiate three successive cycles of selection. The results indicated that there were great reductions in tissue culture parameters (tissue viability, callus initiation, callus proliferation and shoot regeneration) in the three cycles of selection. There were also significant reductions in growth parameters (shoot height, root length, fresh weight and dry weight) of the regenerated plantlets from each of the three cycles. More reduction was induced by the high concentrations. However, after three cycles of selection , the magnitudes of tissue viability, callus initiation, callus proliferation and shoot regeneration were higher in the 3^{rd} cycle relative to the 1^{st} or the 2^{nd} cycle. Thus, it could be concluded from these findings that the inhibitory influence of metribuzin upon tomato growth appeared to be retracted after *in vitro* selection of three successive cycles of growth suggesting an improved resistance of tomato to metribuzin.

The production of improved varieties of plants is central to the long-term viability of agricultural enterprises. Farmers and grazers require new plant varieties to remain financially viable and to be able to farm in a sustainable manner (Dale, 1993). Tissue culture allows the selection of biotypes to be resistant to specific chemical hazards (Smith and Chaleff, 1990; Smith and Derw 1990). Tolerance to herbicides means the survival of the normal population of a species following treatment with a herbicide dosage lethal to other species while resistance means the survival of a segment of the population following treatment with a herbicide dosage lethal to the normal population (Holtum *et al.*, 1994;

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Powles and Holtum, 1994). Tolerant biotypes are only partially affected by the herbicide (Saari *et al.*, 1994; Devine and Preston, 2000).

Metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one] is a systemic herbicide used in soybean (*Glycine max*), potato (*Solanum tuberosum*), tomato (*Lycopersicum esculentum*) and wheat (*Triticum aestivum*). The primary mode of action of metribuzin is inhibition of photosynthesis by binding to a protein in the electron transport system thus effectively blocking energy transport (Corbet, 1994). Many researchers have documented the existence of crop cultivar differential responses to metribuzin treatments (Bahraini-Nejad and Khajehpour, 1999; Lehoczky *et al.*, 2000; Nadasy *et al.*, 2000; Dvořák and Remešová, 2002). Consequently, the present work was aimed to improve the resistance of two cultivars of tomato (*Lycopersicon esculentum* Mill, cv. Karnak vfnt F-1 and Castle rock) to metribuzin herbicide by using tissue culture techniques. Therefore, some growth parameters were checked in normal-grown intact seedlings as well as some tissue culture parameters and growth parameters in *in vitro* selected plantlets.

Material and Methods

Plant materials and growth conditions of normal-grown intact seedlings

Pure seeds of two varieties of tomato (Lycopersicon esculentum Karnak vfnt F-1, KF-1 and Castle rock, CR) were kindly supplied by the Agriculture Research Center, Ministry of Agriculture, Giza, Egypt. Seeds of each variant were planted in potting soil in five 24-cell flats, one flat for a different dose treatment of metribuzin, in a growth room under controlled conditions: (16-h day light, 8-h night, 24/17 °C day night temperature, 450 $\mu Em^{-2}s^{-1}$ PPFD and 75-68% RH). The seedlings were watered with Hoagland nutrient solution as required. Metribuzin was applied to each of the five flats of tomato seedlings at the 4-leaf stage of growth, approximately 28 d after planting. Metribuzin was applied POST at 0, 0.25, 0.5, 0.75, or 1 kg ai ha^{-1}. Two weeks after herbicide treatment, shoots and roots were collected for measurements of growth parameters.

In vitro selection

Cotyledon culture

According to the procedures described by Iler *et al.* (1993), seeds of tomato were surface sterilized for 25 minutes in 25% (v/v) commercial Clorox solution (5.25% sodium hypochlorite) and then rinsed three times in sterile deionized water. The seeds were germinated on Murashige and Skoog (MS) medium, and incubated for three days at 25°C in the dark, then grown under controlled conditions in an incubator (25°C, cool white lights, light intensity 80 $\mu E\ m^{-2}\ s^{-1}$ photo period flux density PPFD, 16 hours light and 8 hours dark) for 4 days. After germination, the cotyledons were excised under aseptic conditions and transferred to a solid selection medium (pH 5.8) containing the nutrients according to Murashige and Skoog (1962), sucrose, 30 g L^{-1}; IAA, 2.63 mg L^{-1} and kinetin, 4 mg L^{-1}. The culture medium was used without or with the simultaneous presence of metribuzin herbicide. A quantity of the herbicide was added to give a final concentration of 0,

0.63, 1.25, 2.5, 5.0 and 10.0 μM metribuzin. Each cotyledon was positioned with its a daxial surface facing upward. Eight 9 cm petri dishes were used for each herbicide concentration and eight cotyledons were cultured per a plate. The tissue was maintained in a growth cabinet at 25°C with a 16-h day and 600 $\mu E\ m^{-2}\ s^{-1}$ PPFD. Shoots were subcultured to metribuzin-free media after 34 days. The cotyledon culture was designated as the first cycle of selection (cycle 1).

Leaf disc culture

Shoots from the untreated control and metribuzin-treated cultures were transferred to solid hormone-free metribuzin-free media in glass jars. Eight of the most vigorous plantlets were selected for further growth on solid hormone-free media in bigger glass jars. After approximately 8 weeks of growth, the youngest fully expanded leaves were excised and used as the source of 0.5 cm diameter leaf discs for the next cycle of selection. A disc from each of the selected plants was cultured per every petri dish on solid media containing the different concentrations of metribuzin.

Eight replicate plates per metribuzin concentration were prepared. The leaf disc medium (pH 5.8) contained the nutrients described by Murashige and Skoog (1962): sucrose, 30 g L^{-1} supplemented with zeatin, 2 mg L^{-1} and naphthalene acetic acid, 0.10 mg. L^{-1} (Behki and Lesley, 1980) or 4 mg BAP [benzyladenine]L^{-1} and 0.2mg IAA L^{-1} (Costa *et al.*, 2000). Leaf discs from a particular plant were placed in the same location on each plate. Forty-eight plates for each variety (eight replicates for each of six herbicide concentrations containing eight leaf discs per plate) were cultured in a growth cabinet under the same conditions as previously described. The first leaf disc culture was designated as the second cycle of selection (cycle 2). Several regenerates were selected from the leaf disc cultures after 2 months of growth and transferred to hormone-free media in large glass jars so that they could be used as donor plants for a third cycle of selection (cycle 3). Leaf discs excised from these plants were cultured on selective media in a manner identical to the second cycle. Counts for tissue viability and callus initiation, as well as callus proliferation ratings, and shoot regeneration frequencies were recorded following each cycle of selection. These values were expressed and compared with the response of the corresponding control tissue. Plants obtained from the three cycles of selection were transferred to soil and cultured in a growth room under controlled conditions (16-h day light, 8-h night, 24/17 °C day night temperature, 450 $\mu Em^{-2}s^{-1}$ PPFD and 75-68% RH).

Plant acclimatization

Acclimatization is a very important step towards the production of normal and mature regenerated plants. Transplanting tomato plantlets from tissue culture media in peat-moss reduced the survival percentage of plants and produced unhealthy plants. Covering plantlets with transparent bags increased the percentage of survival plants and improved plant characters. However, to rescue the regenerated plantlets upon transplantation, they were placed in aquarium condition using Hoagland nutrient solution containing; macronutrients (potassium nitrate 6 mM, calcium nitrate 4 mM, ammonium phosphate 1 mM, magnesium sulphate 2

mM), and micronutrients (boric acid 25 μM, manganese sulphate 20 μM, copper sulphate 0.4 μM, zinc sulphate 0.7 μM, amm. molybdate 0.2 μM). The plantlets were kept in Hoagland solution till they formed a good rooting system (7-10 days) before being transplanting on peat-moss pots (Gafer, 1999). Two weeks after plant transplantation, shoots and roots of the transplanted plants were harvested for determination of growth parameters.

All data of growth parameters and of tissue culture parameters were statistically analyzed using the least significant differences (LSD) method (Snedecor and Cochran, 1980).

Results and Discussion

Intact plant study

Figure 1 shows that metribuzin at all doses used significantly inhibited shoot height and root length of both tomato variants, Karnak vfnt F-1 (KF-1) and Castle rock (CR). The magnitude of inhibition increased with increasing the herbicide dose. The inhibitory effect of the herbicide appeared more greater upon shoot height and root length of CR variant than of KF-1 variant at any dose. The figure also shows similar significant reductions in fresh weight contents of both tomato variants by all doses of metribuzin. The more was the dose of the herbicide, the more was the decrease. The pattern of response was similar for both variants; CR being more affected than KF-1. In the same manner, all doses of the herbicide induced significant reductions in dry weight contents of both variants. The pattern of response was mostly alike in both variants although the magnitude of decrease was usually higher in CR than in KF-1 at all doses. In agreemeint, plant growth was greatly affected by all doses several herbicides. Triazine herbicides were shown to inhibit leaf number, leaf area, fresh and dry weights and photosynthetic activity of several plant species such as tomato (Onofri *et al.*, 1996), wheat (Bahraini-Nejad and Khajehpour, 1999), faba bean (*Vicia faba* L.) (Lehoczky *et al.*, 2000), soybean varieties (Nadasy *et al.*, 2000) and potato varieties (Dvořák and Remešová, 2002).

In vitro selection

As shown in Fig. 2, tissue viability and callus initiation of the two tomato varieties decreased with increasing metribuzin concentration in the growth medium. The pattern of response was mostly similar in the three cycles. The high concentrations of metribuzin (5 μM and 10 μM) induced greater tissue viability and callus initiation in cycle 3 of KF-1 variant than in the other two cycles. Also, more values of callus initiation were found in the 3rd cycle of CR variant by 5 μM and 10 μM metribuzin and of tissue viability by 10 μM.

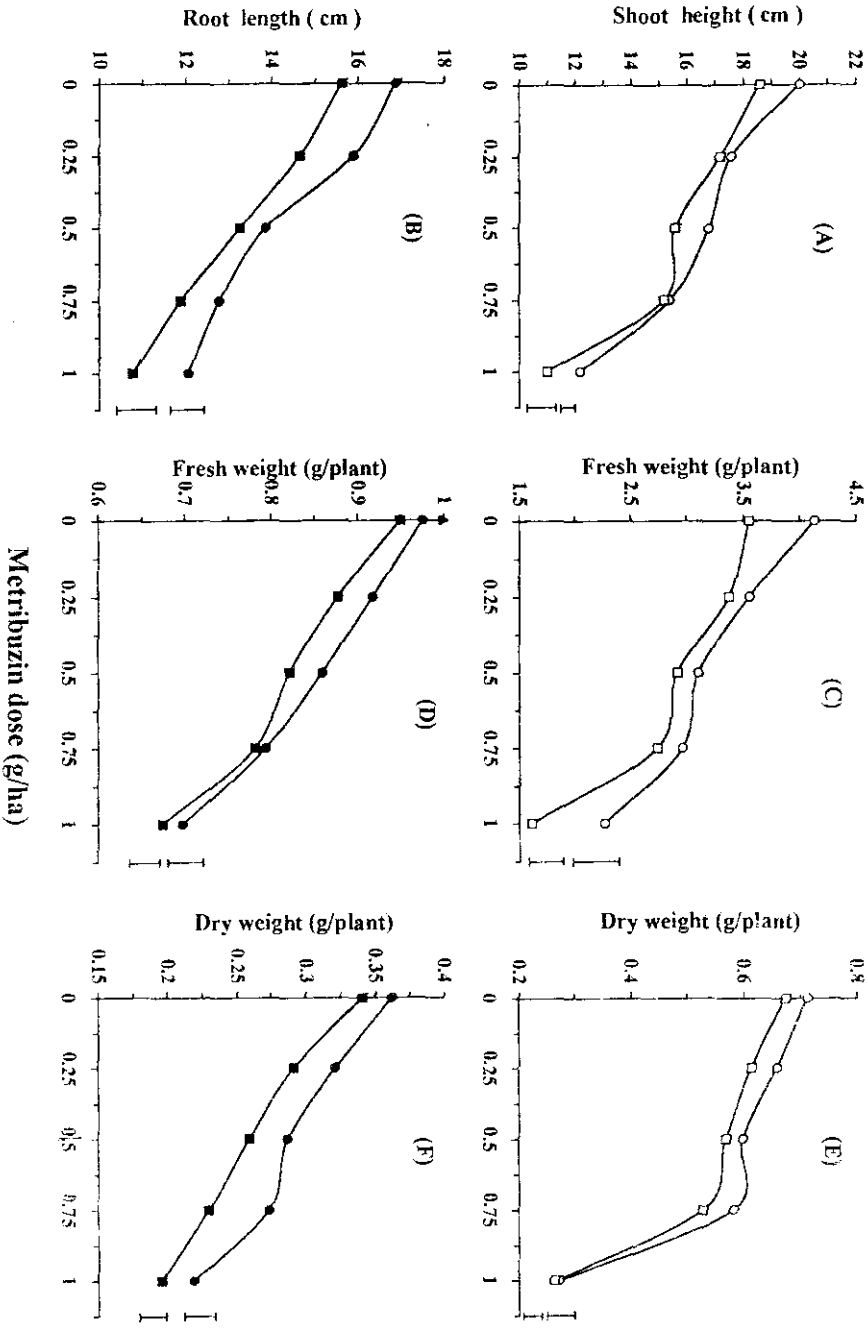

Fig. 1. Effect of metribuzin doses on growth parameters of pot-normally-grown tomato seedlings. ○ and ●, Karnak vfnt F-1 variant; □ and ■, Castle rock variant. Data are means of two experiments. Vertical bars represent LSD values at 5% level.

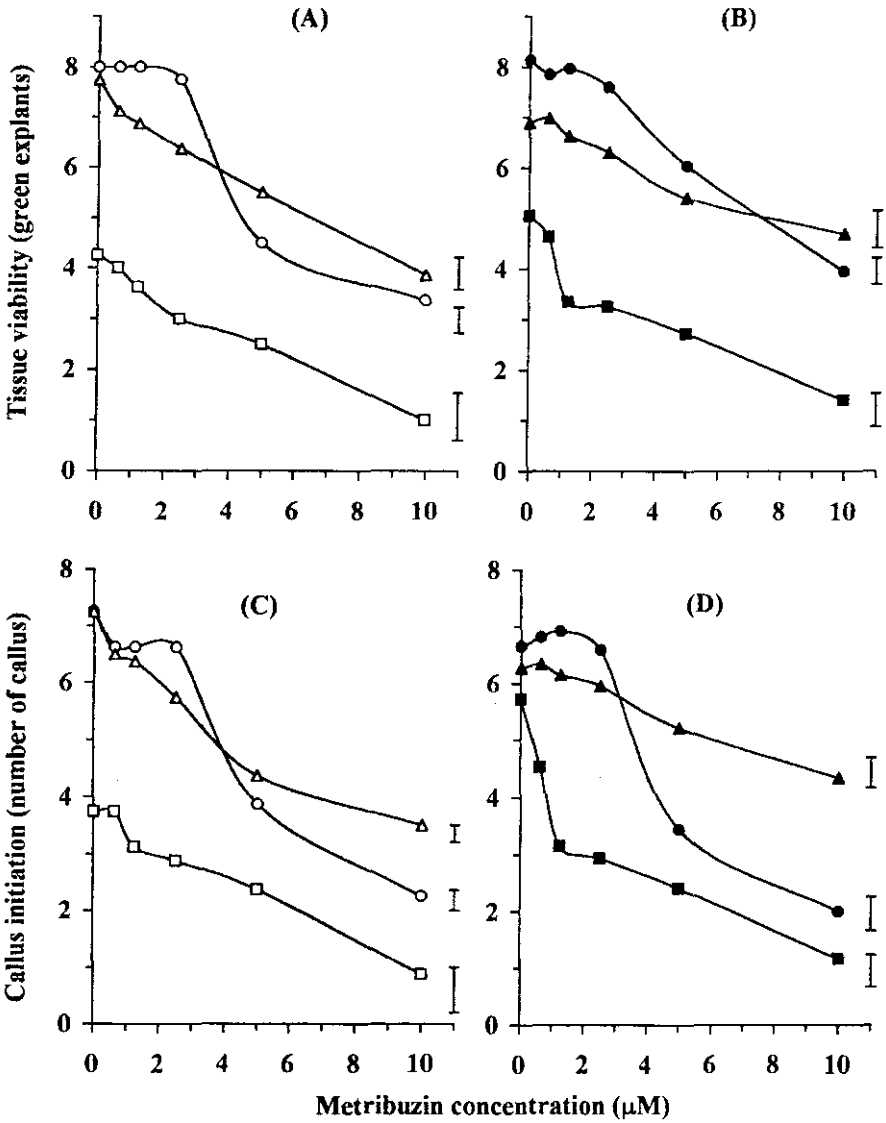

Fig. 2. Effect of metribuzin concentrations on tomato tissue cultures evaluated with respect to tissue viability and callus initiation. Three successive cycles were conducted; ○ and ●, 1st cycle; □ and ■, 2nd cycle; △ and ▲, 3rd cycle. (A) and (C), Karnak vfnt F-1 variant; (B) and (D), Castle rock variant. Data are means of two experiments. Vertical bars represent LSD values at 5% level.

Inspection of Table 1 clearly indicates that there was a retraction in the effect of metribuzin on both viariants of tomato if plants were selected from the 3rd cycle rather than from the 2nd or the 1st cycle. In fact, 10μM of metribuzin reduced tissue viability and callus initiation in both variants more greatly in the 3rd cycle relative to the 1st and the 2nd cycles. In the same pattern, Iler *et al.* (1993) reported that cotyledons and leaf explants from tomato shoots regenerated on media containing imazethapyr were used to initiate three successive cycles of selection. They further observed increases in tissue viability, callus initiation and callus proliferation only following three cycles of selection on 10^{-7}M imazethapyr.

TABLE 1. The percentage of changes, relative to controls, in tissue culture and growth parameters in two tomato variants (*Lycopersicon esculentum* Mill, cv. Karnak vfnt F-1 and Castle rock) variants of tomato as a consequence of treatment with 10 μM metribuzin. KF-1, Karnak vfnt F-1 ; CR, Castle rock; (-), a decrease in the percentage.

Parameters	KF-1			CR		
	Cycles					
	1st	2nd	3rd	1st	2nd	3rd
Tissue viability	-58	-76	-50	-56	-68	-40
Callus initiation	-69	-77	-52	-74	-80	-41
Callus proliferation	-90	-83	-41	-90	-88	-48
Shoot regeneration	-86	-80	-76	-86	-73	-63
Shoot height	-42	-40	-39	-35	-39	-35
Root length	-46	-37	-35	-51	-37	-30
Shoot Fresh weight	-57	-48	-44	-64	-62	-45
Root fresh weight	-66	-68	-62	-67	-67	-62
Shoot dry weight	-80	-68	-66	-80	-68	-62
Root dry weight	-77	-75	-71	-88	-82	-78

In Figure 3, the presence of metribuzin in the growth medium resulted in general decreases in callus proliferation and shoot regeneration in the two tomato varieties Karnak vfnt F-1 and Castle rock. The magnitude of decrease was progressive with the increase in metribuzin concentration. Low concentrations (0.63 μM and 1.25 μM) had no significant effect upon callus proliferation and shoot regeneration of the two variants in all the three cycles. Both concentrations significantly stimulated shoot regeneration from cotyledons in cycle 1 in Castle rock variety. Nevertheless, leaf discs in cycle 2 showed the lowest callus proliferation and shoot regeneration in the treated and untreated tissues of both varieties. Whereas 5 μM and 10 μM metribuzin resulted in an abrupt decline in callus proliferation and shoot regeneration in the 1st cycle but a gradual decrease was found in cycles 2 and 3. Table 1 also shows that 10 μM of metribuzin reduced callus proliferation and shoot regeneration in the 1st cycle by 90% in both variants. This reduction was retracted in the 3rd cycle. In this context,

Sedov *et al.* (1986) indicated that in tissue culture of F1 hybrids of tomato, increasing the concentration of 2,4-D in the medium inhibited regeneration processes and caused morphological changes in the regenerants which were obtained. The preservation of these changes through a callus-plant-callus cycle indicated that they were genetic.

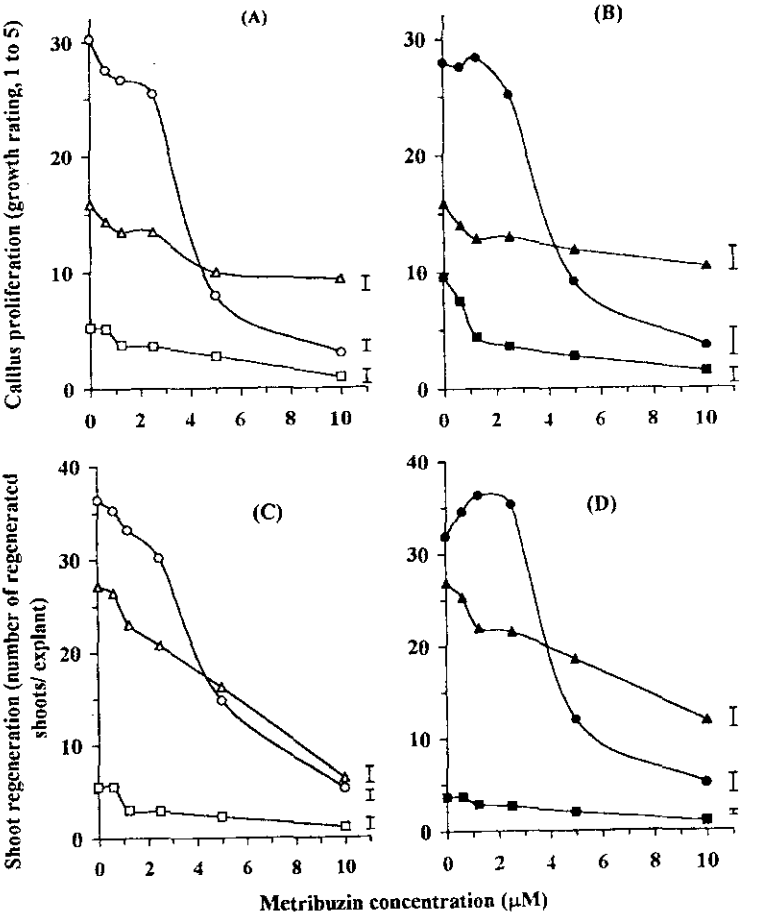

Fig. 3. Effect of metribuzin concentrations on tomato tissue cultures evaluated with respect to callus proliferation and shoot regeneration. Three successive cycles were conducted; ○ and ●, 1st cycle; □ and ■, 2nd cycle; Δ and ▲, 3rd cycle. (A) and (C), Karnak vfnt F-1 variant; (B) and (D), Castle rock variant. Data are means of two experiments. Vertical bars represent LSD values at 5% level.

The shoot height and root length of the *in vitro*- selected plants of both variants were, in general, significantly decreased by metribuzin at all concentration used as compared with the respective untreated tissues although 0.63 μM had no significant effect (Fig.4). The derease in the plant height was progressive with the increase in metribuzin concentration. The effect of metribuzin on shoot height and root length of

plants from *in vitro* selection in cycle 3 was lesser than that of the other two cycles. In accordance, Taregyan *et al.* (2001) found that imazethapyr have an inhibitory effect on root length and shoot dry weight of soybean (*Glycine max*), this inhibitory effect was noted in somaclone seedlings and tissue cultures after selection for resistance to imazethapyr. In confirmation, Standardi *et al.* (1997) concluded that the fresh and dry weights as well as the shoot length were the most severely affected parameters in *in vitro* cultured *Vicia faba* and may be appropriate for preliminary and quick evaluation of herbicide-induced stress.

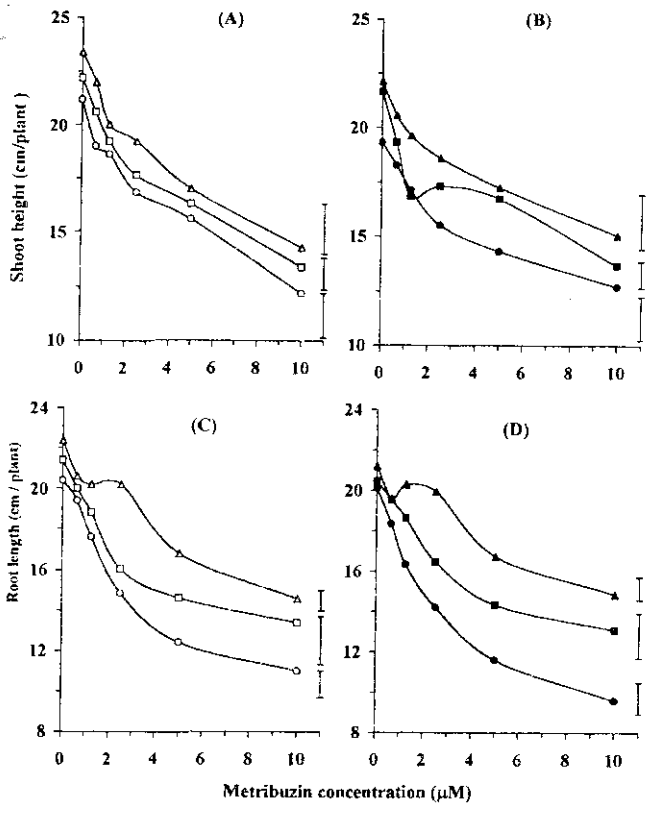

Fig. 4. Effect of metribuzin in the growth medium on shoot height and root length of regenerated plantlets from *in vitro*-grown tomato seedlings during the three successive cycles. ○ and ●, 1st cycle; □ and ■, 2nd cycle; Δ and ▲, 3rd cycle. (A) and (C), Karnak vfnt F-1 variant; (B) and (D), Castle rock variant. Data are means of two experiments. Vertical bars represent LSD values at 5% level.

Figure 5 shows that the presence of metribuzin in the growth medium provoked a significant decrease in fresh weight of shoots and roots of *in vitro*-selected tomato plants as compared with the untreated tissues. The significant decrease was progressive with increasing metribuzin concentrations. Mostly, the effect of metribuzin at all concentrations was greater on fresh weight of shoots

and roots in cycle 1 and cycle 2 plants compared with that of cycle 3 in the two tomato varieties. The higher concentration of the herbicide induced the least effect either on shoot or fresh weight in the 3rd cycle plants as compared with the other two cycles (Table 1).

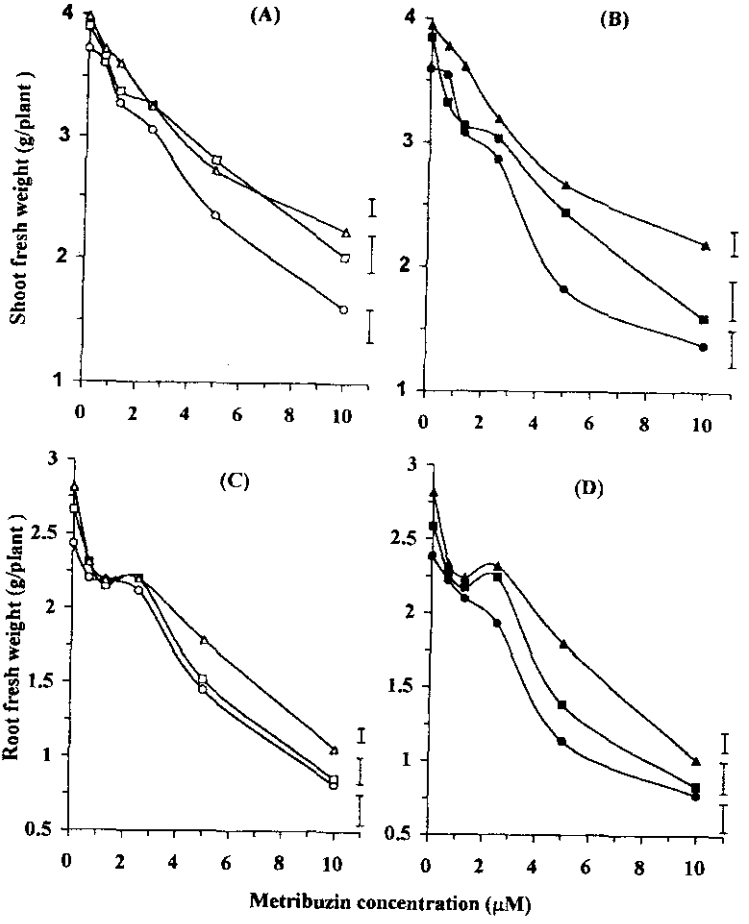

Fig. 5. Effect of metribuzin in the growth medium on shoot and root fresh weight of regenerated plantlets from *in vitro*-grown tomato seedlings during the three successive cycles. ○ and ●, 1st cycle; □ and ■, 2nd cycle; Δ and ▲, 3rd cycle. (A) and (C), Karnak vfnt F-1 variant; (B) and (D), Castle rock variant. Data are means of two experiments. Vertical bars represent LSD values at 5% level.

As observed for fresh weight, dry matter content in shoots and roots of the *in vitro*-selected plants was also significantly affected by metribuzin (Fig. 6). However, there was a significant decrease in shoot and root dry weight of both variants by increasing metribuzin concentration. Plants expressed from the 3rd cycle had a greater dry matter in shoots or roots with respect to the other plants

expressed from the 1st or the 2nd cycles, particularly in Castle rock variety. In accordance, Nemat Alla and Younis (1995) found that shoot height and root length as well as fresh and dry weight of maize (*Zea mays* L.) and soybean (*Glycine max* L.) were generally decreased as a result of treatment with either atrazine or metolachor. In addition, Hassan (2002) detected decreases in dry matter of *Zea mays* due to treatment with glyphosate. On the other hand, Diaz and Marrero (1995) observed no changes in dry matter production of sugarcane varieties treated with ametryn at 1.5, 3, 6, and 12 kg/ha 60 days after shoot formation in the field.

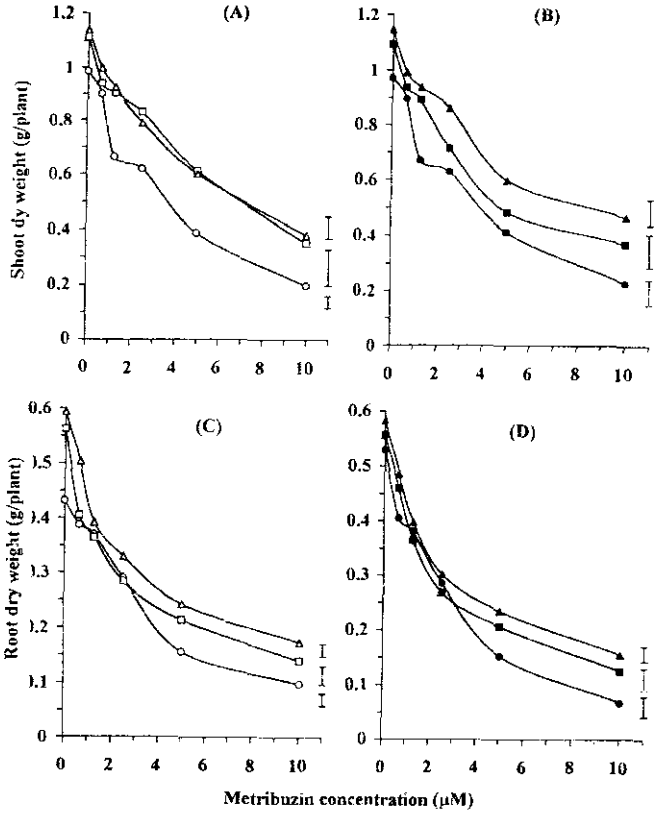

Fig. 6. Effect of metribuzin in the growth medium on shoot and root dry weight of regenerated plantlets from *in vitro*-grown tomato during the three successive cycles. ○ and ●, 1st cycle; □ and ■, 2nd cycle; Δ and ▲, 3rd cycle. (A) and (C), Karnak vfnt F-1 variant; (B) and (D), Castle rock variant. Data are means of two experiments. Vertical bars represent LSD values at 5% level.

Therefore it could be concluded from the present results that both variants of tomato KF-1 and CR are susceptible to metribuzin. This susceptibility is based upon the observed great reduction in growth parameters of both variants by metribuzin. Such influence of the herbicide appeared to be retracted after *in vitro*

selection of three successive cycles of growth. These findings thus might suggest that *in vitro* selection could be considered as an effective technique for improving resistance of tomato to metribuzin.

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استنباط نباتات طماطم مقاومة لمبيد الحشائش ميتربيوزين باستخدام تقنية زراعة الأنسجة. دلائل من قياس معدلات النمو

نعمت محمد حسن ، ممدوح سالم سراج ، أسامة أحمد ممتاز*و محمد محمد الهوارى

قسم النبات – كلية العلوم بدمياط – جامعة المنصورة – دمياط و*معهد بحوث الهندسة الوراثية الزراعية – مركز البحوث الزراعية – الجيزة – مصر .

تم دراسة تأثير مبيد الحشائش ميتربيوزين بجُرَعاتٍ مختلفةٍ (٠,٢٥، ٠,٥، ٠,٧٥، ١,٠ كجم/هكتار) فى الأصص على سلالتين من الطماطم (هجين الكرنك وكاسل روك). أدت المعاملة بالمبيد إلى نقص معنوى فى طول الساق والجذر لكلتا السلالتين وأيضا فى الوزن الطرى والجاف لهما، وخاصة عند استخدام التركيزات العالية من المبيد. لذلك تم إجراء انتخاب نباتات من الطماطم أكثر مقاومة للمبيد باستخدام تقنية زراعة الأنسجة وذلك على ثلاث مراحل متعاقبة من الاسترزاع على بيئات تحتوى على تركيزات مختلفةٍ من المبيد (٠,٦٣، ١,٢٥، ٢,٥، ٥,٠، ١٠,٠ ميكرومولار). وأظهرت النتائج نقصا ملحوظا في حيوية الأنسجة ووجود الكالوس ومعدل نموه وكذلك تمايز السيقان فى كل من المراحل الثلاث وخاصة مع التركيز الأعلى من المبيد. كما نقصت أيضا معدلات النمو (طول الساق والجذر والوزن الطرى والجاف لهما) فى النباتات المنتخبة من الزراعة فى الزجاج من أى من المراحل الثلاث. وكان النقص في هذه القياسات أقل فى حالة النباتات المستنبطة من المرحلة الثالثة عنها فى حالة النباتات المستنبطة من المرحلة الأولى أو الثانية. ويشير هذا التراجع فى التأثير السام للمبيد نتيجة لتعاقب المراحل النامية فى الزجاج إلى اعتبار ذلك زيادة فى مقدرة النباتات المستنبطة بعد ثلاث مراحل من الانتخاب على مقاومة المبيد أو زيادة فى التغلب على تأثيراته السامة.