Egypt. J. Phytopathol., Vol. 32, No. 1-2, pp. 1-10 (2004)

Genetically Resistant Cucumber Plants to Wilt Pathogen *via* Tissue Cultures

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Resistant calli were *in vitro* selected from cucumber explants under challenging stress of cucumber wilt pathogen *Fusarium oxysporum* f.sp. *cucumerinum* culture filtrates (CF). The selection protocol has two directions: First one is step-by-step selection from lower to higher selective CF concentrations; meanwhile the second one is exchangeable continuous cycles with and/or without CF using the same selective CF concentration until the end of selection regime. The progenies of *in vitro* regenerated plants, occurred under CF stress, showed resistance when exposed to the pathogen infection. The results cleared that resistance in cucumber to wilt pathogen is controlled by one pair of genes and segregated as 3 resistant : 1 susceptible. That *in vitro* selective regime *via* tissue cultures is advisable for selection of novel disease-resistant plants.

Key words: Biotechnology, callus, disease-resistance plants, genetics, plant protection, somaclonal variations and tissue cultures.

There is no doubt that the improvement which occurred in descriptive and analytical of the applied and biological sciences has led to modern biotechnology. Thus, new and desirable characters have been introduced and many biological problems are now solved using modern biotechnology. The most important target that ruled the agricultural care allover the world is the pathogenic infections which considered one of the most important problems, which arrested the agricultural economics. However, using the disease-resistant plants cloud solve those pathogenic infections. Many model systems are involved for creating the novel disease-resistant plants. Such model systems ranged from the traditional breeding programs to unconventional modern breeding approaches. Tissue culture techniques as modern unconventional techniques are in use as applicable approaches in different science fields such as plant gene transfer, induction of secondary metabolites, mass propagation, induction of disease resistant or free pathogen plants ...etc.

The success of tissue culture for selecting the novel disease resistant plants depends on what so called 'somaclonal variations'. Additionally, tissue culture is considered a bio-safely approach because it utilize the genetic variability that occur naturally and independently in the cellular genetic material of the host plant giving genetically mosaics' tissues (Evans, 1984 and 1989; Buiatti *et al.*, 1985 and EL-Kazzaz and Malepszy, 1994). Such somaclonal variations offer an opportunity for the breeders to generate and select specific variants (Malepszy and EL-Kazzaz, 1990; EL-Kazzaz and Malepszy, 1994; EL-Kazzaz, 1995; EL-Kazzaz, 2001 and EL-Kazzaz and EL-Mougy, 2001).

Many researchers revealed the utilization of somaclonal variations that coupled with the pathogen culture filtrate or its toxins for selecting the novel disease resistant plants (Buiatti *et al.*, 1985; Chawla and Wenzel, 1987 a&b; Lamb *et al.*, 1989; Crino *et al.*, 1996; EL-Kazzaz and Abdel-Kader, 1998 and El-Kazzaz *et al.*, 1999; EL-Kazzaz, 2001 and EL-Kazzaz and EL-Mougy, 2001).

Thus, this investigation was planned to select and develop cucumber resistant plants from selected resistant callus variants to wilt disease caused by *Fusarium oxysporum* f.sp. *cucumerinum*. The genetic background of the selected cucumber resistant plants was also investigated.

Materials and Methods

The Polish pickling cucumber (*Cucumis sativus* lv. Borszczagowski) was used in this study. Tested cucumber seeds were soaked for two hr. in sterilized distilled water with little drops of liquid soap. These seeds were surface sterilized by immersing in 20% sodium hypochlorite containing 5.5% active chlorine for 30 minutes, washed thoroughly in four changes of distilled sterilized water, then left between two folds of sterilized filter papers for the excess water to be removed. Sterilized seeds were individually cultivated, under aseptic conditions, on 100 ml modified MS medium (Murashige and Skoog, 1962), which was used as inducing-callus medium in 250 ml jars. The modified MS medium was composed of half strength of macro- and micro-elements, vitamins, 1.5% sucrose and 0.75% agar. The pH was adjusted to 5.8 before autoclaving. The jars were incubated in a dark culture room at 25-28°C until root initiation, then incubated under *c.* 2000 Lux of fluorescent lamps with 16 hr light : 8 hr dark at the same temperature for 3 weeks. Calli were induced from explants of cucumber plants according to EL-Kazzaz (1996). The induced calli were re-cultivated for mass production for further studies.

Fusarium oxysporum f.sp. *cucumerinum* causing wilt in cucumber plants was isolated from infected plants (EL-Kazzaz, 1995) and used as pathogen system in the presented study. Tested cucumber plants were highly infested by the isolated pathogen. The pathogen was cultured on two kinds of media. First medium was Potato Dextrose Agar (PDA), which was used as a solid medium for interval subculturing of the fungus. The second medium was Richard's nutrient (R) (Heitefuss *et al.*, 1960), which was used as a liquid medium for producing the fungus culture filtrate. The pH of these media was adjusted to 4.0.

Good growing fungal colonies on PDA medium were transferred to the liquid (R) medium for 3 weeks of culture in the dark at 25-28°C. The cultures were then sieved by nylon sieves and sterilized twice by bio-filtration using double 45µ filter membranes. Five litres of (CF), per each pathogen system, were collected and conserved at (-20°C) in a deep freezer for further studies.

Various concentrations of culture filtrate (CF) were poured into MS medium. Equal amounts of water from MS medium were replaced by the same amount of stock (CF) to give concentrations of 5% to 100% of the selective agent (CF)

measured to the medium volume. Three controls were prepared as follows: two controls of MS medium supplemented with 5% or 50% of (R) medium instead of equal amounts of water from MS medium and the third one was MS0 as MS medium without (CF) or (R).

Selection protocol:

The selection protocol was performed with the callus cultures on MS medium contains various concentrations of the culture filtrate (CF) of the aforementioned pathogen. Such protocol was initially targeted in two directions as shown in Fig. (1).

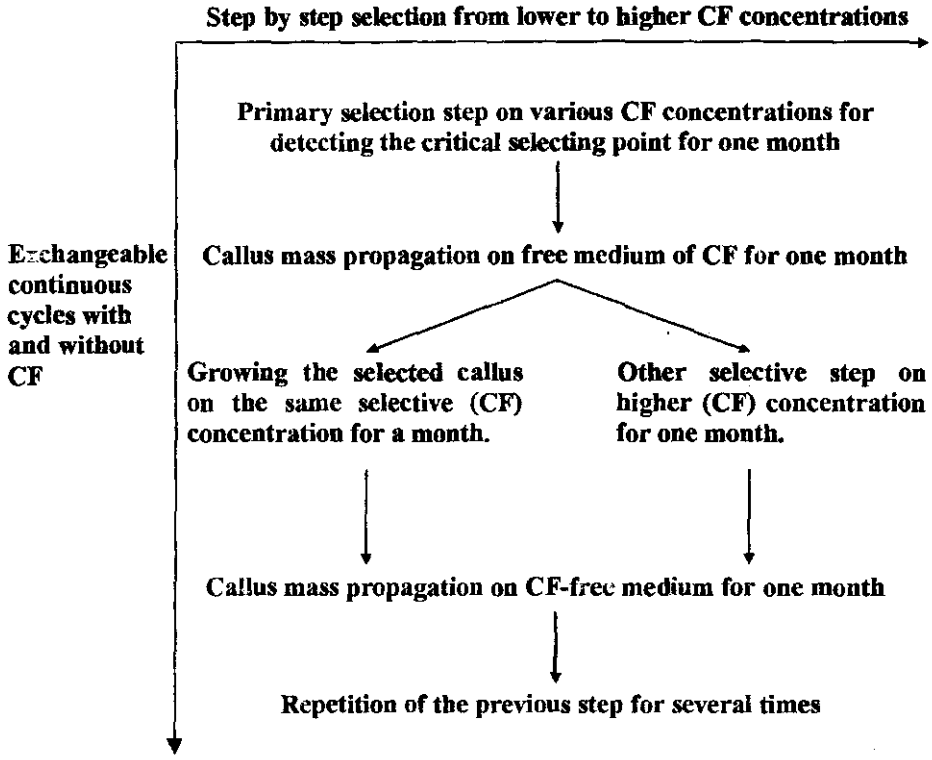

Fig. 1. Scheme of selection protocol used *in vitro* for selecting cucumber calius variants on selective (CF) of *Fusarium oxysporum* f.sp. *cucumerinum*.

The obtained calli were cultured on a selective MS medium supplemented with 5% to 100% of (CF) for selection system to estimate the critical concentration of the selective agent. The survived calli under stress were exposed to other cycle of selection, which contains the same and the higher concentrations of the selective agent.

The selected callus cultures, which showed stable resistance during the selection process, as well as their controls, were aseptically transferred onto MS medium for regeneration process as descried by EL-Kazzaz and Malepszy (1994). The regenerated plants were cultivated in the greenhouse for seed production.

Pathogenicity test:

Cucumber plants were evaluated for susceptibility to the tested pathogen under greenhouse conditions in pots containing loamy soil infested with the pathogen at the rate of 5% (w/w). The percentage of infected plants was calculated 60 days after transplanting. The plantlets, regenerated from selected calli, were evaluated for their resistance to 100% of pathogen CF under laboratory conditions.

The selected regenerates and the original genotypes were transplanted in pots under greenhouse for getting their seeds. Such pots were filled with disinfected loamy soil. The cultivated next generation plants were irrigated with the pathogen suspension ($5x10^7$ spores/ml) at the rate of 250 ml/pot every 2 days. The percentage of pathogen incidence was calculated four weeks after transplanting.

Resluts and Discussion

Tested cucumber plants recorded 80-90% susceptibility to *Fusarium oxysporum* f.sp. *cucumerinum* after 60 days of infection under greenhouse conditions.

Data in Table (1) show that at the first selection cycle, the crucial concentration of *F. oxysporum* f.sp. *cucumerinum* (CF) was 15% whereas 250 sectors from callus cultures were isolated with little growth. The higher dosages of (CF) were killers for the callus tissues. The massed selected sector tissues were grown on CF-free medium for one month. Meanwhile, 67 selected callus colonies out of the pervious 250 colonies tolerated 25% (CF) as higher concentration. The selected colonies were grown on CF-free medium for one month subcultures.

Table 1. Selection cycles of cucumber callus colonies and regenerates occurred on media supplemented with different (CF) concentrations of *Fusarium oxysporum* f.sp. *cucumerinum*

Experiment	Selection cycles of cucumber callus with CF								
	5 cycles on 15%			4 cycles on 25%			3 cycles on 50%		
	T *	S	R	T	S	R	T	S	R
Exp.1	560	115	36	115	25	8	25	20	5
Exp.2	550	65	22	65	22	5	22	19	4
Exp.3	370	70	24	70	20	4	20	17	3
Total	1480	250	82 **A**	250	67	17 **B**	67	56	12 **C**

* T= Tested, S= Selected and R= Regenerated.

The massed calli were grown and stressed again with higher (CF) concentrations as a third selection cycle. However, 56 callus colonies could be isolated on medium with 50% (CF). Obtained results are concurring with those recorded by many researchers (Arcioni *et al.*, 1987; Buiatti *et al.*, 1985; Chawla and Wenzel, 1987a&b; El-Kazzaz, 1995; El-Kazzaz, 1996 and El-Kazzaz and Abdel-Kader, 1998). Moreover, El-Kazzaz *et al.* (1999) stated that such variations may be attributed to the induction of mutations (somaclonal mutations) within the callus cells. However, the selected calli regenerated on media amended with 15%, 25% and 50% of (CF), gave 82, 17 and 12 selected regenerates, respectively, and designated as groups A, B and C.

Data in Table (2) reveal that after 96 hr of continuous exposure to 100% CF, the seedlings from group A, B and C gave 10, 12 and 11 good growing seedlings out of 82, 17 and 12 tested plants, respectively. Data in Table (2) clear also that the selected regenerates of group A, B and C, which showed the tolerance to CF, represented 12.20%, 70.59% and 91.73%, respectively, and reached 29.73% of the total tested plants. That test was evolved to ensure about the stability of the developing seedlings to resist the pathogen toxins and the other excreted substrates (Agrios, 1989; Lamb *et al.*, 1989; El-Kazzaz and Malepszy 1994). Such resistance frequently results in activation of plant defense responses (Hammond-Kosack and Jones, 1997).

Table 2. Selection of regenerates on 100% CF and ($*x10^7$) spores /ml of fungal infection of *Fusarium oxysporum* f.sp. *cucumerinum*, respectively

Group designation	No. of regenerates after 69 hr of exposure on 100%CF			Response of regenerates to fungal infection			
	Tested	Selected	(%)	R *	(%)	S	(%)
A	82	10	12.20	4	40.00	6	60.00
B	17	12	70.59	5	41.67	7	58.33
C	12	11	91.67	5	45.45	6	54.55
Total	111	33	29.73	14	42.42	19	57.58

* R= Resistant plants and S= Susceptible plants.

These results are in harmony with those recorded by Evans (1989); El-Kazzaz (1995); Crino *et al.* (1996); El-Kazzaz (1996); El-Kazzaz *et al.* (1999); El-Kazzaz (2001) and El-Kazzaz and EL-Mougy (2001) that gave explanation of reasons why the use of CF could give selected resistant plants to all mechanisms of host-pathogen interaction according to gene-for-gene theory. This test also referred that the genetic background might still have heterogeneous gene structures for resistance to toxins of *F. oxysporum* f.sp. *cucumerinum*.

Furthermore, the previous regenerated plants were intensively grown on strong concentration ($5x10^7$) of the pathogen suspension, which was added every two days under greenhouse conditions. The tested regenerates from group A, B and C recorded 40.00%, 41.67% and 45.45% of resistant individuals, respectively, and

reached 42.42% of the total tested plants (Table 2). On the other hand, tested plants gave sensitive individuals, recorded 60.00%, 58.33% and 54.55%, in the three groups, respectively, and represented an average of 57.58% of the total tested plants. Such results predicated that tested plants probably genetically gave one resistant individual : one sensitive individual ratio according to Mendelian's background.

However, such previous prediction was proved with next generations using Chi-square (χ^2) test as shown in Table (3). Chi-square (χ^2) per each tested group or for all the groups was highly significant and below the tabled Chi-square (χ^2= 3.841; 5.412 or 6.635) with the probability 0.05; 0.02 or 0.01 at one degree of freedom, respectively. These results give a prophecy that resistance to *F. oxysporum* f.sp. *cucumerinum* in cucumber may be controlled by one pair of genes either homozygous or heterozygous dominant for conferring such resistance giving the value of 3 resistant : 1 susceptible or 1 resistant : 1 susceptible ratio. Such results agree with the findings of El-Kazzaz and Malepszy (1994) and El-Kazzaz (1995) who studied the *in vitro* resistance of cucumber plants to *F. oxysporum* f.sp. *cucumerinum.*

Table 3. Genetic factors segregated with 1Resistant : 1 Susceptible or 3 Resistant: 1 Susceptible ratio of tested plant phenotypes by *Fusarium oxysporum* f.sp. *cucumerinum* and proved with Chi-Square (χ^2) test

Tested group	Kind of * plants	No. of seeds	Infection incidence and Chi-square (χ^2) value				
			Resistant	Sensitive	Resistant (%)	Sensitive (%)	χ^2
A	$C_{♀}$ X $R_{♂}$	40	16	24	40.00	60.00	1.600
	$R_{♀}$ X $C_{♂}$	35	14	21	40.00	60.00	1.400
	$R_{♀}$ X $R_{♂}$	42	28	14	66.66	33.34	1.554
	$R_{♀}$ X $R_{♂}$	43	29	14	67.44	32.53	1.309
B	$C_{♀}$ X $R_{♂}$	70	32	38	45.70	54.30	0.690
	$R_{♀}$ X $C_{♂}$	75	33	42	44.00	56.00	1.080
	$R_{♀}$ X $R_{♂}$	80	63	17	78.75	21.25	0.600
	$R_{♀}$ X $R_{♂}$	50	35	15	70.00	30.00	0.666
	$R_{♀}$ X $R_{♂}$	40	27	13	67.50	32.50	1.200
C	$C_{♀}$ X $R_{♂}$	52	29	23	55.77	44.23	0.692
	$R_{♀}$ X $C_{♂}$	60	27	33	45.00	55.00	0.600
	$R_{♀}$ X $R_{♂}$	44	30	14	48.18	31.82	1.094
	$R_{♀}$ X $R_{♂}$	54	37	17	68.51	31.49	1.200
	$R_{♀}$ X $R_{♂}$	75	54	21	72.00	28.00	0.337

* $C_{♀}$ or $C_{♂}$ = control plants and $R_{♀}$ or $R_{♂}$ = Regenerates.
- Degrees of freedom= 1. Probability at 0.05= 3.841 & at 0.02= 5.412 and at 0.01= 6.635.

In conclusion, the obtained results cleared that the occurred cellular genetic variability's are created as somatic (somaclonal) mutations within the callus cells

giving mosaics of resistant and sensitive cells (Arcioni *et al.*, 1987; Chawla and Wenzel, 1987 a&b; El-Kazzaz, 1996 and El-Kazzaz and Abdel-Kader, 1998). Moreover, using cycles of selection on medium, with and/or without the pathogen CF, lead to produce calli not adapted to the CF but tolerant to CF that will decrease such mosaic cells and help only the resistant cells to generate on the selective medium. However, using the exchangeable cycles of selections on medium, with or without CF, evolved to ensure the resistance stability of the getting cultures or plants (Agrios, 1989; Lamb *et al.*, 1989; Malepszy and El-Kazzaz, 1990; El-Kazzaz and Malepszy 1994; El-Kazzaz, 1995; Crino *et al.*, 1996; El-Kazzaz, 1996; El-Kazzaz *et al.*, 1999; El-Kazzaz, 2001 and El-Kazzaz and EL-Mougy, 2001).

Additionally, using the higher CF concentration may give a chance to eliminate the sensitive cells. These results support those reported by Malepszy and EL-Kazzaz (1990); El-Kazzaz (1995) and EL-Kazzaz and Abdel-Kader (1998). It is worth to affirm that resistance to CF might be due to mutations in suppressive genes that were accompanying the normal genes of resistance in the background of the callus cells.

However, Hammond-Kosack and Jones (1997) stated that cell death is often induced by toxins and/or enzymes excreted by the pathogen and targeted to specific substrates. They also referred that resistance can be achieved *via* the loss or alteration of the toxin's target or through detoxification. This fact declares that selected tissues might alter to act with *F. oxysporum* f.sp. *cucumerinum* and its toxins by inducing toxin suppressers or detoxification enzymes. Therefore, using such selection protocol may give a chance to eliminate the sensitive cells (Malepszy and El-Kazzaz, 1990 and El-Kazzaz and Malepszy, 1994). These findings may explain the reasons of why the use of CF could give selected resistant plants to all mechanisms of host-pathogen interactions according to gene-for-gene theory. Such resistance frequently results in activation of plant defense responses (Hammond-Kosack and Jones, 1997).

Finally, presented research considers one of the most effective methods for inducing the resistance in plants. Whereas, it is bio-safely method, low rate costing, not leprous, not need big space for work and faster than the traditional breeding programs about 2-3 folds.

References

Agrios, G.N. 1989. *Plant Pathology.* 3rd Ed., Academic Press, California, USA. Pp. 63-155.

Arcioni, S.; Pezzotti, M. and Damiani, F. 1987. *In vitro* selection of alfalfa plants resistant to *Fusarium oxysporum* f.sp. *medicaginis*. *Theor. Appl. Genet.*, **74**: 700-705.

Buiatti, M.; Scala, A.; Bettini, P.; Nascari, G.; Morpugo, R.; Bogani, P.; Pellegrini, G.; Gimelli, F. and Veturo, R. 1985. Correlations between *in vitro* resistance to *Fusarium* and *in vitro* response to fungal elicitors and toxic substances in carnation. *Theor. Appl. Genet.*, **70**: 42-47.

Chawla, H.S. and Wenzel, G. 1987a. *In vitro* selection of barley and wheat for resistance against *Helminthosporium sativum*. *Theor. Appl. Genet.*, **74**: 841-845.

Chawla, H.S. and Wenzel, G. 1987b. *In vitro* selection for fusaric acid resistant barley plants. *Plant Breeding*, **99**: 159-163.

Crino, P.; Cristnzio, G.; Gentile, A.; Haegi, A.; Rugini, E. and Soressi, G.P. 1996. Culture filtrates and toxins in the selection of disease resistant plants. Proc. of the Workshop: Plant Breeding for Resistance to Biotic Stresses: Physiological and Molecular Basses, Monsampolo Del Toronto, Italy, 19-20 May 1994. *Petria, 1996, 6: Supplemento*, **1**: 197-217.

EL-Kazzaz, A.A. 1995. *In vitro* stability of disease resistance to *Fusarium oxysporum* in F_3 cucumber plants *via* tissue culture. *Egypt. J. Appl. Sci.*, **10** (9): 640-655.

EL-Kazzaz, A. 1996. Transmission of inherited somaclonal variations in F_3 cucumber plants to its regenerated plants *via* callus cultures. *Egypt. J. Appl. Sci.*, **11** (3): 304-321.

EL-Kazzaz, A.A. 2001. Inheritance of resistance to *Fusarium oxysporum* f.sp. *lycopersici* in F_2 tomato plants *via* tissue culture. *The Egypt. J. of Genetics and Cytology*, **30** (1):51-59.

EL-Kazzaz, A.A. and Abdel-Kader, M.M. 1998. *In vitro* selection of tomato callus culture and regenerated plants on culture filtrate of *Fusarium oxysporum* f.sp. *lycopersici*. *Egypt. J. Phytopathol.*, **26** (1): 37-50.

EL-Kazzaz, A.A.; Abdel-Kader, M.M; Hussein, M.H. and Hussein, H.A. 1999. Molecular markers for somaclonal variants of faba beans resistant to root-rot (*Fusarium solani*). *Arab. J. Biotechnol.*, **2** (2):167-180.

EL-Kazzaz, A.A. and EL-Mougy, Nehal S. 2001. Inheritance of disease resistance in cucumber plants to root rot caused by *Fusarium solani* using tissue culture techniques. *Egypt. J. Phytopathol.*, **29** (2): 57-68.

EL-Kazzaz, A.A. and Malepszy, S. 1994. Selection of resistant *Cucumis sativus* regenerated plants to *Fusarium oxysporum* via tissue culture. Proc. of the 1st Egyptian - Italian Sympos. on Biotechnol., Assiut, Egypt. Pp. 121-130.

Evans, D.A. 1984. Genetic basis of somaclonal variation in tomato. Pages: 259-265. In: International Symposium of Plant Tissue and Cell Culture Application to Crop Improvement. Prague, Czechoslovakia.

Evans, D.A. 1989. Somaclonal variation-genetic basis and breeding applications. *Trends in Genetics*, **5**: 46-50.

Hammond-Kosack, K.E. and Jones, J.D.G. 1997. Plant disease resistance genes. *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, **48**: 575-607.

Heitefuss, R.; Stahmann, M.A. and Walker, J.C. 1960. Production of pectolytic enzyme and fusaric acid by *Fusarium oxysporum* f.sp .*gonglutinass* in relation to cabbage yellow. *Phytopathology*, **50**: 367-370.

Lamb, C.J.; Lawton, M.A.; Dron, M. and Dixon, R.A. 1989. Signals and transduction mechanisms for activation of plant defences against microbial attack. *Cell*, **56**: 215-224.

Malepszy, S. and EL-Kazzaz, A. 1990. *In vitro* culture of *Cucumis sativus*. XI. Selection of resistance to *Fusarium oxysporum*. *Acta Horticulturae*, **280**: 455-458.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**: 473-497.

(Received 13/01/2004;
in revised form 10/03/2004)

نباتات خيار مقاومة وراثيا لمسبب الـذبول باسـتخدام المزارع النسيجية

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تم الحصول على كالوسات (calli) مقاومة ومنتخبة معمليا من مستقطعات نباتية من الخيار في وجود (صفر- ١٠٠%) من راشح الفطر المسبب لمـرض الذبول في الخيار *Fusarium oxysporum* f.sp. *cucumerinum*. وفي هذا الصدد تم استخدام نظام انتخابي من اتجاهين: الاتجـاه الأول يعتمـد علـى انتخاب الكالوس بنظام الخطوة بخطوة (step-by-step) على مـادة الانتخـاب حيث يبدأ من التركيز الأقل إلى التركيز الأعلى لراشح الفطر. والاتجاه الثـاني يعتمد على نمو الكالوس المنتخب على دورة متغيرة مستمرة على بيئات خاليـة من عامل الانتخاب (راشح الفطر) ثم النمو على بيئات بها عامل الانتخاب حتى نهاية الدورة الانتخابية.

هذا وقد أوضحت الدراسة أن المستولدات النباتية يمكن أن تتكون تحت تأثير عامل الحفز الانتخابي وأن هذه المستولدات أظهرت المقاومة عندما تعرضـت للإصابة المباشرة بالمسببات المرضـية المـستخدمة. وقـد أظهـرت أجيـال المستولدات النباتية مقاومتها للفطر المسبب لمرض ذبـول الخيـار المـستخدم وانعزلت وراثيا بنسبة ٣ مقاوم : ١ غير مقاوم.

هذا النظام الانتخابي المعملي باستخدام المزارع النسيجية يمكـن التوصـية بتطبيقه بغرض انتخاب نباتات جديدة مقاومة للإصابات المرضية حيث يتميـز بكونه مقتصد للوقت والمكان وغير مكلف ماديا كما أنه آمن حيويا.