Selection of *Pseudomonas solanacearum*-Resistant Tomato Plants *via* Tissue Culture

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(Received October 9, 2005)

ABSTRACT: *For in-vitro selection of wilt-resistant tomato, effect of different concentrations of P. solanacearum-culture filtrate (CF) on callus induction and shoot regeneration of two tomato cultivars (Castle Rock and Super Strain B) was examined. For both cultivars, a regular declining in the mean fresh weight of calli, derived from cotyledonary leaf explants, was observed with increasing CF concentration. Treating explants of Castle Rock and Super Strain B cultivars with 20% of CF showed highly toxic effect as they decreased the mean fresh weight of their developed calli to 38 and 35%, respectively. Moreover, treating explants of Castle Rock and Super Strain B cultivars with 15% of CF decreased the percentage of explants producing shoots to 20.5 and 21.7%, respectively. However, increasing CF to 20% has significantly decreased the percentage of explants producing shoots of Castle Rock and Super Strain B cultivars to 7.6 and 10.2%, respectively. From each cultivar, 20 CF-resistant regenerants developed on medium containing 20 % CF were successfully obtained. These regenerants were evaluated for their reaction to the inoculation with the virulent culture of Pseudomonas in greenhouse. Of the 20 plants regenerated from the Castle Rock cultivar that were selected on 20% CF, 5 plants showed no wilting while the rest plants showed a moderate welting after 3-4 weeks of infection and did not produce fruits. Seven plants regenerated from Super Strain B on shoot regeneration medium supplemented with 20% of the culture filtrate were the most resistant as they showed no wilting and produced fruits.*

INTRODUCTION

Exploitation of the variation that exists in populations has led to the development of many commercial varieties and hybrids. With the rapid expansion of tissue culture technologies came the observation that genetic variation was occurring in plants regenerated from somatic cells and this was seen as a novel source of variation (Larkin and Scowcroft, 1981). The potential of somaclonal variation involves the ability to change one or a few characters without altering the remaining part of a genotype. The frequency of off-types varies with species, culture type and number of sub-cultures and has been attributed to a number of variations within cultured cells (Scowcroft *et al.*, 1987). These changes are expected to generate stable plants carrying interesting heritable traits. For plant breeders, this variation may be an advantage as selection for new traits can be carried out among the regenerated population. In this case, there is no need to use artificial mutagensis as uncontrolled variability occurs spontaneously and in large enough numbers to select for different characteristics within the regenerated plants (Cresswell, 1991).

As a result of somaclonal variation, a wide range of plant characteristics can be altered. However, the selection of agronomically important traits, e.g. disease resistance, has many limitations. The efficiency of selection can be increased by the application of *in vitro* selection procedures (Van den Bulk, 1991). *In vitro* selection involves the screening of cell cultures that are exhibiting genetic variation, for tolerance or resistance to pathogens, herbicides, low or high temperatures, metals and salt (Tomes and Swanson, 1982 and Chaleff, 1983). Initial reports of potentially useful variation included increases in cane and sugar yield, and resistance to eye-spot disease in sugar cane (Heinz *et al.*, 1977); improved tuber shape, colour, and late blight resistance in potato; and, increased solids and Fusarium resistance in tomato (Evans and Sharp, 1983 and Evans, 1989).

The use of *in vitro* screening to select for disease resistance is more effective for diseases in which the pathogen produce toxin(s). This was initially demonstrated by Carlson (1973) who regenerated tobacco plants resistant to *Pseudomonas tabaci*, from haploid cells resistant to methionine sulfoxamine, an analogue of the disease toxin. Since then, the induction of disease-resistant plants has been successfully developed in several diseases such as southern leaf blight of maize caused by *Helminthosporium maydis* race T (Gengenbach

and Green, 1975 and Gengenbach *et al.*, 1977) and early blight of potato caused by Alternaria solani (Matern *et al.*, 1978). Ishida and Kumashiro (1988) reported that, application of brown spot disease (*Alternaria alternaria*) toxin to cultured cells or protoplasts of tobacco might be an efficient method for evaluating the degree of resistance of tobacco lines. An application of culture filtrates of pathogens may be also valuable for the selection of resistant cells to cell wall degrading enzymes as well as toxins.

Bacterial wilt caused by the soil-borne bacteria *Pseudomonas solanacearum* is found in most of the tropical, sub-tropical and temperate countries. Among the wide range of hosts of this bacteria, tomato is susceptible to suffer severe losses. Bacterial wilt-resistant tomato plants were obtained using a tomato tissue culture system (Toyoda *et al.*, 1989). The toxin responsible for causing wilt disease in tomato was isolated from *Pseudomonas solanacearum* race 1 biovar 3 by Baruah and Deka (1995). Cell lines of 5 wilt susceptible tomato varieties, growing in modified MS medium, were treated with purified toxin and cells showing toxin insensitivity were isolated and regenerated to plants. One regenerated somaclone showed a high level of resistance to *P. solanacearum*, while two other somaclones showed moderate resistance. Field tests of the somaclones for 3 years showed segregation of the character.

The objective of the present study is to establish an efficient *in vitro* selection system to select for bacterial wilt-resistant tomato, under the local Egyptian conditions, through tissue culture.

MATERIALS AND METHODS

Plant cultivars and bacterial strains

Virulent isolate of *P. solanacearum* isolated from wilted plants in the main Solanaceae growing areas of Minia, Egypt was used. It was kindly provided by Dr. A. Galal, Department of Plant Pathology, Faculty of Agriculture, Minia University, Egypt. Two commercial varieties of tomatoes (*Lycopersicon esculentum* Mill.) namely, Castle Rock and Super Strain B, which are known to be sensitive to *P. solanacearum*, were used in this study

Preparation of bacterial culture filtrate

Bacterial strain was shake-cultured at 260C in PCG medium (10 g Bacto-peptone, 10 g casamino acid, and 10 g glucose in 1 L of distilled water, pH 6.8) for 10 days. Crude culture was subjected to membrane filtration then, kept frozen until using.

Callus Culture and Plant regeneration

Seeds were surface sterilized and germinated on growth regulators-free MS medium (Murashige and Skoog, 1962) supplemented with 30 g sucrose/liter as described by Mahmoud *et al.*, 2004. For callus culture, cotyledonary leaves were excised from the 14 days old seedling and transferred to MS_2 medium (Mahmoud *et al.*, 2004). For selection of resistant calli, MS_2 medium containing 0.0, 5, 10, 15 and 20% CF was used. Eight replicates, each replicate involved 5 jars and each jar involved 5 explants, were set. After transferring the explants to these media the vials were incubated at 25 ±2 °C under a 16/8 h photoperiodic regime. After one month of incubation, the mean fresh weight of calli/replicate developed on MS_2 of both genotypes (Castle Rock and Super Strain B) was determined.

For shoot regeneration, cotyledonary leaves were cultured on MS_1 medium (Mahmoud *et al.*, 2004) supplemented with 0.0, 5, 10, 15 and 20% CF. Eight replicates, each replicate involved 5 jars and each jar involved 4 explants, were set. After 21 days of incubation at 25 ±2 °C under a 16/8 h photoperiodic regime, the mean number of explants produced shoots/replicate were determined.

Some of the developed shoots were excised and transferred to the rooting medium containing MS salts, MS vitamin mixture, 3% (w/v) sucrose, without growth regulators, pH 5.8 and solidified with 0.8% agar. Well-developed leaflets were acclimatized as described by Mahmoud *et al.*, (2004). These plants were grown in a greenhouse for three weeks and inoculated with *P. solanacearum*.

Inoculation with virulent strain of *P. solanacearum*

The regenerated plants were inoculated with *P. solanacearum* by the method previously reported (Toyoda *et al.*, 1989). Plant roots growing in soil were injured with a razor blade, at positions 5 cm below soil surface and 2 cm distant from stems. Ten ml of bacterial suspension (10^8 cells/ml) of *P. solanacearum* was poured directly onto injured roots. Inoculated plants were grown in the greenhouse at 28^0C, and bacterial wilt damage, expressed as percentage of wilted plants, was evaluated 3 weeks after inoculation.

RESULTS AND DISCUSSION

Effect of culture filtrate on callus induction

Lethal effects of the culture filtrate (CF) was examined by culturing cotyledonary leaf explants on callus induction medium (MS_2) supplemented with different concentrations of CF. The mean fresh weight of CF-treated explants as well as the non-treated explants are shown in Table (1). No detectable changes in callus tissue were observed when explants were cultured on CF-free medium. However, for both cultivars, treatment with culture filtrate showed callus browning and detectable deterioration of callus growth. The results showed that, increasing CF concentration was accompanied with gradual declining of the mean fresh weigh of calli. Treating explants of Castle Rock and Super Strain B cultivars with 20% of CF was highly toxic as they decreased the mean fresh weight of calli to 38 and 35%, respectively. These results suggest the release of toxic substances by virulent bacteria which significantly affect the growth of calli.

Similarly, selection for resistance against toxin produced by Alternaria alternaria genotypes was obtained at 30% toxin concentration which causes a 90 % reduction in callus growth of sunflower (Kintzios *et al.*, 1996). After one month in culture, 18% of the callus demonstrated resistance to the toxin. Behnke (1979) showed that the culture filtrate of *Phytophthora infestans* was functional for the selection of resistant potato callus to the toxin occurring in the filtrate. Similar successes have been reported with a range of diseases including *Phoma lingam* in canola (Sacristan 1982), *Helminthosporium oryzae* in rice (Vidhyasekaran *et al.*, 1990). El-Kazzaz and Abdel-Kader (1998) selected *in vitro* resistant calli of three tomato genotypes under challenging of culture filtrates that obtained from wilt pathogen.

Table (1): The mean fresh weight of explants after 1 month of culturing on MS_2 medium supplemented with culture filtrate.

Cultivars	Culture Filtrate (B)				
(A)	0.0	5%	10%	15%	20%
Castle	6.25	5.35	5.03	3.90	2.36
Rock	(100)	(86)	(80)	(62)	(38)
Super	7.84	7.05	5.71	4.12	2.71
strain B	(100)	(90)	(73)	(53)	(35)
Mean	7.05	6.20	5.37	4.01	2.53

Data are means of 8 replicates (each replicate included 5 jars of 5 explants).
LSD (A), at 0.05 = 0.238 at 0.01 = 0.316
LSD (AXB), at 0.05 = 0.337 at 0.01 = 0.447

Effect of culture filtrate on shoot regeneration

Lethal effects of the culture filtrate on shoot regeneration were examined by culturing cotyledonary leaf explants on shoot regeneration medium (MS_1) supplemented with different concentrations of CF. The mean numbers of explants that could produce shoots are shown in Table (2). No significant changes in the number of explants producing shoots were observed when explants were cultured on medium containing 5% CF comparing to explants cultured on CF-free medium. However, for both cultivars, about 50% of explants could produce shoots in the presence of 10% culture filtrate. Treating explants of Castle Rock and Super Strain B cultivars with 15% of CF decreased the percentage of explants produceding shoots to 20.5 and 21.7 %, respectively. However, increasing CF to 20% significantly decreased the percentage of explants which could produce shoots of Castle Rock and Super Strain B cultivars to 7.6 and 10.2 %, respectively. These results suggest a highly significant effect of CF on the regeneration of shoots from both cultivares of tomato used in the present experiment.

Table (2): The main numbers of explants which could produce shoots after 1 month of culturing on MS_1 medium supplemented with culture filtrate.

CF (%)	Cultivars	
	Castle Rock	Super Strain B
0.0	18.25 a (100.0%)	19.62 a (100.0%)
5	17.13 a (93.9%)	18.50 a (94.3%)
10	9.25 b (50.7%)	10.75 b (54.8%)
15	3.75 c (20.5%)	4.25 c (21.7%)
20	1.38 d (7.6%)	2.00 d (10.2%)
$LSD_{0.05}$	**1.54**	**1.37**

Data are means of 8 replicates (each replicate contained 5 jars of 4 explants [a])
[a] Means followed by the same letter in a column are not significantly different at $P < 0.05$.

Shoots regenerated on medium containing 20% CF were transferred to the selective medium for root formation. Finally, 20 CF-resistant regenerants were successfully obtained from CF-treated explants of each cultivar.

In comparable study, various concentrations of culture filtrates from Fusarium subglutinans were tested for their

effect on *in vitro* multiplication of plantlets and regeneration from calli of resistant and susceptible pineapple (Hidalgo *et al.*, 1999). The culture filtrate reduced shoot formation of *in vitro* plantlets and regeneration of plants from calli derived from the susceptible cultivars. However, shoot formation and regeneration of plants from calli derived from the resistant plants were not affected. These results show the possibility of using culture filtrates of F. subglutinans to provide a method of in vitro screening of pineapple for resistance to the pathogen.

Inoculation of CF-resistant regenerants with virulent bacteria.

Forty regenerants from each cultivar, 20 CF-resistant and 20 regenerated on CF-free medium, were rooted and acclimatized. The acclimatized plantlets were transferred to wider pots. Regenerated plants of the two tomato cultivars were evaluated for their reaction to *Pseudomonas* in a greenhouse following inoculation. Inoculated control plants showed a partial yellowing of lower leaves 4-6 days after inoculation and subsequently partial wilting in upper leaves of plants. As a result, all control plants were completely wilted within 15 days after inoculation. On the other hand, CF-resistant regenerants did not show such symptoms at this early stage of infection.

Regarding the Castle Rock, among the 20 plants regenerated on 20% CF, 5 showed no wilting while the other plants showed a moderate welting after 4 weeks of infection and could not produce fruits. Seven plants regenerated from Super Strain B on MS_1 medium supplemented with 20% of the culture filtrate were the most resistant as they showed no wilting and could produce fruits. Fruits of the resistant plants were harvested and their seeds were collected for studying the inheritance of resistance in the subsequent generations.

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انتخاب نباتات طماطم مقاومة لبكتريا البسيدومونس سولانسريم عن طريق مزارع الأنسجة

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تم في هذه الدراسة اختبار تأثير راشح المزارع البكتيرية لبكتريا البسيدومونس سولانسريم علي تكون الكالوسات وتكشف الأفرع الخضرية لصنفين من الطماطم (كاسل روك ، سوبر أسترين بى) وذلك بزراعة الأوراق الفلقية والتي استخدمت كمنفصلات نباتية على بيئات تحتوى علي تركيزات مختلفة من راشح المزارع البكتيرية. ولكلا الصنفين كانت الزيادة في تركيز الراشح البكتيري مصحوبة بنقص متزايد في متوسط وزن الكالوسات الناتجة على بيئة النمو. وكان لإضافة 20% من الراشح البكتيري تأثير عالى السمية حيث أن هذا التركيز أدى إلى انخفاض متوسط الوزن الطازج للكالوسات إلى 38% ، 35 % للصنفين كاسل روك ، سوبر أسترين بى على التوالى وذلك بالمقارنة بالكنترول (الكالوسات المتكونة على البيئة الخالية من الراشح البكتيري). وزراعة المنفصلات النباتية على البيئة المحتوية على 15% من الراشح البكتيري أدى إلى أنخفاض نسبة المنفصلات النباتية التى أمكنها تكوين أفرع خضرية إلى 20.5 و 21.7 للصنفين كاسل روك وسوبر أسترين بى على التوالي. وبزيادة تركيز الراشح البكتيري إلى 20% حدث انخفاض معنوى في نسبة المنفصلات النباتية التي أمكنها تكوين أفرع خضرية إلى 7.6 و 10.2% للصنفين.

وامكن في هذه الدراسة الحصول علي 20 نبات مقاوم للتركيز المرتفع من الراشح البكتيري من كل صنف من الصنفين وتم اختبار استجابة هذه النباتات للعدوى ببكتريا البسيدومونس سولانسريم في الصوبة. فبالنسبة للصنف كاسل روك كانت هناك 5 (خمس نباتات) فقط من العشرين نبات المختبرة لم تظهر عليها أعراض الإصابة (الذبول) واستمرت في النمو وأمكنها تكوين ثمار أما باقي النباتات (15) حدث لها ذبول جزئى بعد 3-4 أسابيع من العدوى ولم تكون ثمار . وبالنسبة للصنف الآخر (سوبر أسترين بى) فإن 7 (سبع نباتات) فقط من العشرين نبات المختبرة كانت أكثر مقاومة حيث لم تظهر عليها أعراض الإصابة واستمرت إلى مرحلة تكوين الثمار علي العكس من باقي النباتات التي حدث لها ذبول جزئي نتيجة العدوى ولم تكون ثمار .